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Characterization of four septin genes, and detection of genetic interactions between *WdCDC10* and chitin synthase genes during yeast budding in the polymorphic mold, *Wangiella* (*Exophiala*) *dermatitidis*.

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by

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Dissertation

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Dedication

This work is dedicated to my LORD, **God**. I also dedicate this work to my family without whom I would not have had the strength and support, which is necessary to succeed throughout my graduate work.

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I am sure that no words are sufficient to express my appreciation to Dr. Szaniszlo. I profoundly thank Dr. Szaniszlo for providing me the chance to study the field of fungal biology. Above all, I really appreciate his understanding and support for me all throughout the years. I also would like to thank my committee, Dr. Makkuni Jayaram, Dr. Arturo De Lozanne, Dr. Scott W. Stevens, and Dr. Alan M. Lloyd, for very helpful and constructive suggestions on my project. My thanks to Dr. Jungsuk Kang for pRS vectors and Dr. John R. Pringle for *S. cerevisiae* mutant strains. I also acknowledge everybody including the former and current members of the Szaniszlo Lab, especially Dr. Hongbo Liu, Dr. Dariusz Abramczyk, Pangfei Guo, and Tuan Vo for their help and support. I also appreciate the encouragement from my friends in the Payne lab, especially Megan Boulette and Stephanie Craig, and the many Korean students in the biology programs. Finally, special thanks to my family again.

Characterization of four septin genes, and detection of genetic interactions between *WdCDC10* and chitin synthase genes during yeast budding in the polymorphic mold, *Wangiella* (*Exophiala*) *dermatitidis*.

Publication No._____

Changwon Park, Ph.D.

The University of Texas at Austin, 2007

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Septins are a highly conserved family of eukaryotic proteins having significant homology within and among species. In the budding yeast, *Saccharomyces cerevisiae*, a septin-based hierarchy of proteins is required to localize chitin in the bud neck prior to septum formation. However, this process has not been clarified in a filamentous, conidiogenous fungus capable of yeast growth, such as *Wangiella dermatitidis*, a polymorphic agent of human phaeohyphomycosis. Prior studies of this melanized mold showed that some chitin synthase mutants (*wdchs*Δ) have defects in yeast septum formation, suggesting that the septins of *W. dermatitidis* might functionally associate with some of its chitin synthases (WdChsp). To test this hypothesis, four vegetative septin homologs of *S. cerevisiae* were cloned from *W. dermatitidis* and designated *WdCDC3*, *WdCDC10*, *WdCDC11*, and *WdCDC12*. Of the four, only *WdCDC3* functionally complemented completely a strain of *S. cerevisiae* with a ts mutation in the

corresponding gene, although *WdCDC12* did so partially. Functional characterizations by mutagenesis of the four *W. dermatitidis* septin genes revealed that resulting mutants (*wdcdc*Δ) each had unique defects in yeast growth and morphology, indicating that each septin carried out a distinct function. Furthermore, when a *wdcdc10*Δ mutation was introduced into five different *wdchs*Δ strains, weak genetic interactions were detected between *WdCDC10* and *WdCHS3* and *WdCHS4*, and a strong interaction between *WdCDC10* and *WdCHS5*. Cytological studies showed that WdChs5p was mislocalized in some septin mutants, including *wdcdc10*Δ. These results confirmed that in *W. dermatitidis* septins are important for proper cellular morphogenesis, cytokinesis, and especially septum formation through associations with some chitin synthases.

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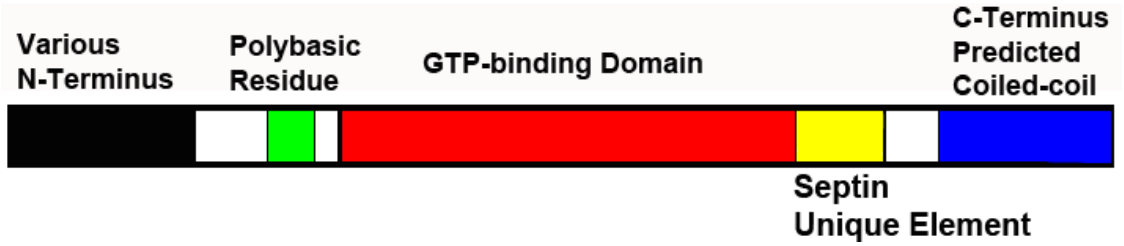
I. INTRODUCTION

1. THE PRIMARY STRUCTURE OF THE SEPTINS

Approximately 30 years ago, the septins were originally identified because they were found to be defective in certain temperature-sensitive (ts) mutants that control the cell division cycle in the yeast *Saccharomyces cerevisiae*. The *cdc3*, *cdc10*, *cdc11*, and *cdc12* ts mutant strains displayed cell cycle arrest and similar defects in cytokinesis or cell separation at the restrictive temperature, which resulted in multiply-budded cells with elongated and multiply-nucleated buds (Hartwell, 1971). Septins are present in eukaryotes ranging from fungi to mammals. Interestingly, an aquatic green alga, *Chlamydomonas reinhardtii* encodes *bona fide* septin orthologs (Grossman *et al*., 2003), but higher plants seem to lack septins.

Sequence analysis has revealed that septins have about 30% overall amino acid sequence identity within and among species and consist of an N-terminus polybasic region, a GTPbinding domain, and with exceptions of Cdc10p orthologs, a predicted coiled–coil domain at their C-terminus (Figure 1). The N-terminus polybasic region is thought to be required for binding to phosphoinositides in membranes, as shown for the basic domain in the mammalian septin H5 (Zhang *et al*., 1999). The GTP-binding domain of a septin is flanked on the N-terminus side by a short polybasic motif and on the C-terminus side by a conserved domain or element that is unique to the septin protein family. The GTP-binding domain itself contains the

Figure 1. The motifs and domains of the septins. The GTP-binding domain (red) contains septin-unique elements (yellow). The predicted coiled-coil sequence in the C-terminus extension (blue) is absent in Cdc10p orthologs.



characteristic p-loops [G1 (GXXXXGKT), G3 (DTPG), and G4 (XKXD)] that are conserved in other GTPase domains, but which are not highly homologous to those of the well-known Ras family of GTPases (Leipe *et al*., 2000). Interestingly, however, not all individual septins appear to function as GTPases. When present, the C-terminus coiled-coil region seems to work together with the adjacent conserved domain to bind to other septins or proteins (Versele *et al*., 2004). Both *in vitro* and *in vivo*, septins form filamentous heteromeric septin-septin complexes (Casamayor and Synder, 2002; Field *et al*., 1996) that bind and hydrolyze GTP. However, some results have suggested that septin filament formation does not require associated GTP hydrolysis (Mitchison and Field, 2002) and that instead the GTP binding and/or hydrolysis of GTP is important for interactions between septins and non-septin proteins (Casamayor and Synder, 2002) or phospholipids (Zhang *et al*., 1999). Thus, the specific role of GTP binding and hydrolysis by septins is currently still poorly understood.

1. 1 Septins in *Saccharomyces cerevisiae*

Septins have been most widely studied in *S. cerevisiae*, which has seven septins: Cdc3p, Cdc10p, Cdc11p, Cdc12p, Shs1p/Sep7p, Spr3p, and Spr28. During sporulation, Spr3p and Spr28p, which only function in sporulation (Ozsarac *et al*., 1995; De Virgilio *et al*. 1996), co-localize with Cdc3p, Cdc10p, and Cdc11p at the edge of the forespore (Longtine and Bi, 2003). However, the exact mechanism and function of the septins in sporulation remains unclear. During vegetative growth, five septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p/Sep7p) are co-expressed and co-

localized (Byers *et al*., 1976). These so-called vegetative septins form a pentameric complex that is not formed in the absence of any septin protein, except of Shs1p/Sep7p. Apparently, this septin, which is not required for filament complex formation, serves to enhance this structure's stability and to act as a target for regulation by phosphorylation (Dobbelaere *et al*., 2003). Cdc3p, Cdc11p, and Cdc12p are believed to form the main filament polymer, while Cdc10p acts as a bridge protein to promote cross-linking between the filaments (Figure 2).

Deletion analysis in *S. cerevisiae* has proven that *CDC3* and *CDC12* are essential genes for viability and that *cdc10* and *cdc11* mutants show cytokinesis defects and aberrant morphologies, such as elongated, multinucleated buds (Longtine *et al*., 2000; Lee *et al*., 2002). In contrast, the *shs1/sep7* mutant cells exhibit only very mild defects in cytokinesis and budding (Mino *et al*., 1998).

Shortly (~15 min) before yeast bud emergence in *S. cerevisiae*, a 10-nm filamentous septin ring forms at the site of bud emergence (Ford and Pringle, 1991). As the yeast cell cycle proceeds, the septin ring expands into the emerging daughter bud and forms the so-called "collar," which is an hourglass-shaped structure at the mother-bud neck cortex. While the collar structure is stable during the G_2, S, and M phases with the onset of cytokinesis, the septin ring structure splits into two distinct rings as the actomyosin ring starts to contract (Figure 3; Ford and Pringle, 1991). Finally, after cell separation, the cortical septin rings remain for a short period at the site of cell division, and are then degraded by sumoylation mediated by an E3-ligase-like Siz1p by conjugation of Smt3p, which is required for proper disassembly of septin complexes (Johnson and Gupa, 2001).

Figure 2. Model for the septin complex structural organization. Each septin, as a dimmer, associates with other septins. One Cdc3p-Cdc10p-Cdc12p and one Cdc3p-Cdc10p-Cdc11p dimer pair then, form a complex, with Cdc10p acting as a bridge. Shs1p/Sep7p is thought to bind to Cdc11p through the coiled-coil region of Cdc11p (modified from Versele and Thorner, 2005).

Figure 2

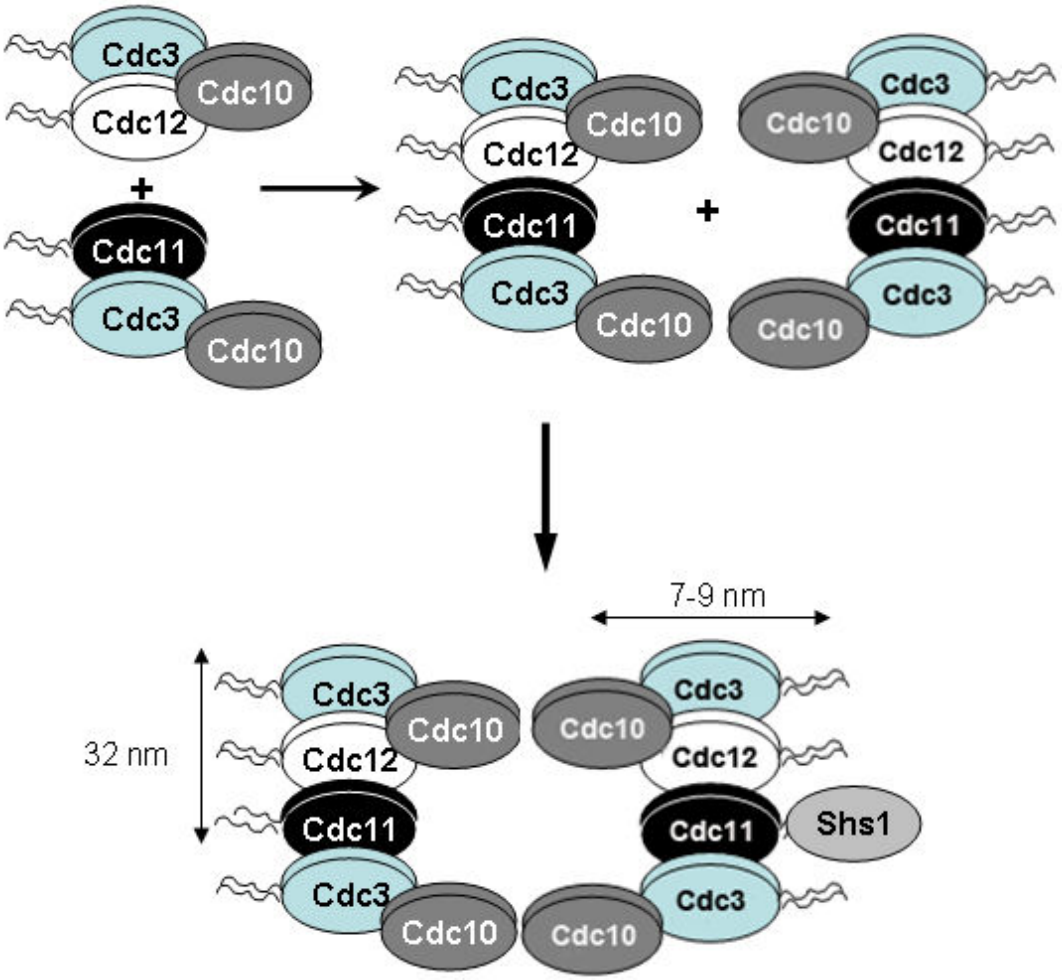
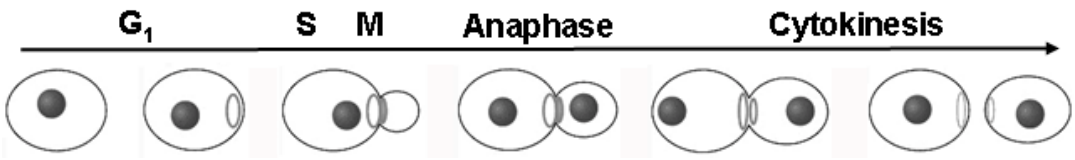

Cdc3
Cdc10
Cdc12
Cdc11
Cdc3
Cdc10
Cdc3
Cdc10
Cdc12
Cdc11
Cdc3
Cdc10
Cdc10
Cdc3
Cdc12
Cdc11
Cdc3
Cdc10
7-9 nm
32 nm
Cdc3
Cdc10
Cdc12
Cdc11
Cdc3
Cdc10
Cdc10
Cdc3
Cdc12
Cdc11
Shs1
Cdc3
Cdc10

Figure 3. Septin localization during *S. cerevisiae* cell cycle progression. At the late G_1 phase, septins (in red) localize at the presumptive bud site about 15 min before a bud emerges. Through S and M phases, the septin ring expands into the daughter cell and is stabilized. After the nucleus (in blue) divides and at cytokinesis, the septin ring splits into two distinct rings. After cell separation, the cortical septin rings remain at the site of cell division and eventually are degraded by sumoylation (modified from Longtine and Bi, 2003).

Figure 3



Two models for septin function have been suggested in *S. cerevisiae*. In one, the scaffold model, septins act as a 'template' for recruiting other proteins responsible for cytokinesis. The other is known as the diffusion-barrier model and functions during G_2-M transition.

1. 1. 1 The scaffold model of septin function.

In the "scaffold model," septins are proposed to form a structure at the mother-bud neck and to act as a "template" for recruiting other proteins involved in various cellular processes. Among the proteins reported to be recruited by the septin proteins of yeast are Myo1p for actomyosin contractile ring formation and cytokinesis (Bi, 2001), Bud3 and Bud4 for bud site selection and emergence (Casamayor and Synder, 2002), and Chs2p and Chs3p for chitin deposition (Schmidt *et al*., 2002; De Marini *et al*., 1997), all of which play critical roles in polarity and cellular morphogenesis (Longtine and Bi, 2003).

1. 1. 1. 1 Bud site selection.

Proteins related to bud site selection in *S. cerevisiae* are good examples of some proteins recruited by septins. There are two bud site selection patterns. In the first, haploid cells produce a new bud adjacent to the previous cell separation site; this is the "axial" budding pattern (Chant and Pringle, 1995), for which Bud3p (Chant *et al*., 1995), Bud4 (Sanders and Herskowitz, 1996), and Axl2p/Bud10p (Casamayor and Synder, 2002) need to be recruited to the bud neck. In the "bipolar" budding pattern, a bud emerges near the poles in diploid cells (Chant and Pringle, 1995). To achieve the

bipolar budding pattern, correct localizations of Bud8p, together with actin, the formin Bni1p (Chant and Pringle, 1995), Bud9p, and the integral membrane proteins Rax1p and Rax2p (Kang *et al*., 2001) are required. For both axial and bipolar budding, Bud5p, a guanine-nucleotide exchange factor (GEF) and Bud2p, a GTPase activating protein (GAP), also need to be recruited to the septin rings (Kang *et al*., 2001; Park *et al*., 1999).

1. 1. 1. 2 Bud morphologenesis checkpoint and sensors.

In *S. cerevisiae*, Hsl1p (a protein kinase), Hsl7p (a protein arginine methyltransferase), and Swe1p (a *S. cerevisiae* homolog of Wee1p, Booher *et al*., 1993) a cyclin-dependent kinase (CDK)-inhibitory kinase), are recruited to the septin collar, thereby localizing at the bud neck (Longtine *et al*., 2000; Shulewitz *et al*., 1999). Among these proteins, Swe1p is a critical component for the bud morphogenesis checkpoint, although it is dispensable for other cell cycle checkpoints. When the bud morphogenesis checkpoint is triggered by an inappropriate bud size or other stresses, it introduces a G_2 delay in the nuclear cycle, which allows time for further bud growth before nuclear division (Lew and Reed, 1995). In the G_2/M phase transition, Hsl1p and its binding partner, Hsl7p co-localize with Swe1p at the daughter side of the mother bud neck and they are required for Swe1p localization and degradation (Lew and Reed, 1995). However, localization of these proteins is lost in septin ts mutant stain backgrounds (Longtine *et al*., 2000). Moreover, subsequent studies have proven that Cdc3p physically interacts with Hsl1p (Barrel *et al*., 1999) that in turn interacts physically with Hsl7p, which finally interacts directly with

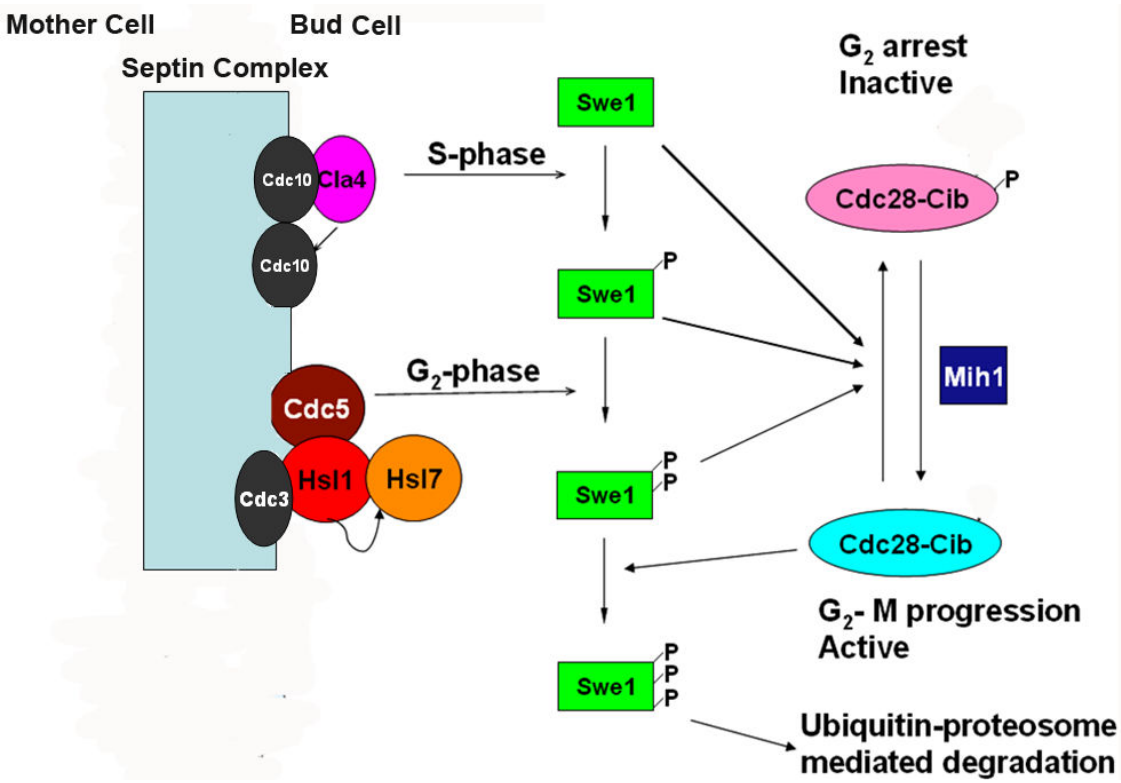
Swe1p (Shulewitz *et al*. 1999). Collectively, these data suggest that a septin hierarchy of protein recruitment is essential for the morphogenesis cell cycle checkpoint (see Figure 4). However, as with many results related to septins, their exact relationships to the mechanisms and functions of septins on other cell cycle checkpoints are still unclear.

1. 1. 2 The diffusion barrier model of septin function.

There are two excellent pieces of evidence that support the "diffusion barrier" model of septin function. The first is the asymmetrical localization of green fluorescent protein (GFP)-tagged Ist2p. In wild type (WT), the GFP-Ist2p in the plasma membrane of the bud neck on the daughter cell side is unable to relocate to the mother side. However, in the *cdc12-6* ts mutant background, when the septin ring is perturbed by high temperature, the GFP-Ist2p is observed instead at the mother-side of the bud neck (Takizawa *et al*., 2000). The second relates to Lte1p, the GEF of Tem1p GTPase. In WT cells, Lte1p-3GFP (three tandem copy of GFP) functions normally and localizes at the bud cortex and is not found in mother cells. However in *cdc10*Δ cells, Lte1p-3GFP is mobile and diffuses into mother cells (Castillon *et al*., 2003). In spite of these results, it remains unclear how the septins are anchored in the plasma membrane and how the septin-mediated diffusion barrier is formed at the bud neck.

Figure 4. The septin hierarchy of protein recruitment of the morphogenesis checkpoint. Septin complexes give a template for the localization of the Hsl1p-Hsl7p-Swe1p complex to the bud neck. Cdc3p recruits Hsl1p, which physically interacts with its binding partner Hsl7p, which in turn recruits Swe1p to the daughter side of mother bud neck (modified from Versele and Thorner, 2005).

Figure 4


Mother Cell
Bud Cell
Septin Complex
Cdc10
Cla4
Cdc10
S-phase
G2-phase
Cdc5
Cdc3
Hsl1
Hsl7
Swe1
Swe1
P
Swe1
P
P
Swe1
P
P
P
G2 arrest
Inactive
Cdc28-Cib
P
Mih1
Cdc28-Cib
G2- M progression
Active
Ubiquitin-proteosome
mediated degradation

1. 1. 3 The function of *S. cerevisiae* septins in sporulation.

Under nutrient limitation conditions, *S. cerevisiae* cells develop four haploid spores called ascospores, in a cell called an ascus. Because the spores are derived by meiosis of a diploid zygote nucleus, ascospores are considered to be sexual spores. The sporulation process is very different from that of normal yeast budding, since there is no cell separation site or bud neck. Instead, the spore membranes are newly synthesized progressively around the postmeiotic nuclei.

During late meiosis of the sporulation process, the transcription levels of *CDC3*, *CDC10*, and the two sporulation-specific septin genes, *SPR3* and *SPR28*, are increased more than 10-fold from those of vegetative growth (Chu *et al*., 1998). Later, the septins, Cdc3p, Cdc11p, Spr3p, and Spr28p form ring-like structures around each of four spindle pole bodies. However, these ring-like structures are different from those that form during vegetative growth, because they initially localize at the leading edge of the prospore membrane and then expand as the prospore membrane expands in order to enclose each meiotic nucleus. Generally, during the sporulation process, septins are thought to co-localize with each other in prospore membranes and form a complex, because the localization of Cdc3p and Cdc11p is affected in the *spr3*Δ strains (Fares *et al*., 1996). However, further studies are required to gain a more profound understanding of the regulations and interactions between septins and other proteins during sexual sporulation.

1. 2 Septins in *Candida albicans*.

To date, seven septins (CaCdc3p, CaCdc10p, CaCdc11p, CaCdc12p, CaShs1p/CaSep7p, CaSpr3p, and CaSpr28) also have been identified in *C. albicans*, a dimorphic fungus that grows as yeast or hyphae. The amino acid sequence identities to those of *S. cerevisiae* are between 33% (the lowest, CaSpr28p) and 69% (the highest, CaCdc10p). Moreover, as in *S. cerevisiae*, *CaCDC3* and *CaCDC12* are essential for viability, whereas strains lacking *CaCDC10* or *CaCDC11* are viable. However, these mutants show defects in yeast development (*i.e.* cytokinesis defects), similar to those of *S. cerevisiae cdc10* and *cdc11* mutants, respectively. In addition, *cacdc10* and *cacdc11* mutants also show severe morphological defects in hyphae, such as abnormal shapes and irregular cell wall deposition patterns, as compared with WT (Warenda and Konopka, 2002). Interestingly, *cacdc10* and *cacdc11* mutants are less invasive both *in vitro* and *in vivo* and are less virulent in mouse models of acute infection (Warenda *et al*., 2003).

In contrast to *S. cerevisiae*, septin ring formation was observed in *cacdc11*Δ yeast cells even at 42 °C (Warenda and Konopka, 2002). This observation contradicts the current models, which suggest that Cdc11p has an important role in septin filament formation (An *et al*., 2004). Although the *C. albicans* septins appear also to act as a scaffold to recruit proteins to the bud neck, *C. albicans* seems to lack Hsl7p and Hsl1p, which play a significant role in morphogenesis checkpoint in the *S. cerevisiae* (Longtine *et al*., 2000). Moreover, *C. albicans* septins appear not to be modified by Siz1p-mediated sumoylation (Smt3p), as are *S. cerevisiae* septins (Johnson and Gupa, 2001). Nonetheless, the overall localization pattern of septins during yeast budding

(Warenda and Konopka, 2002) and pseudohyphae formation (Sudbery *et al*., 2004) in *C. albicans* is similar to that in *S. cerevisiae* (Ford and Pringle, 1991).

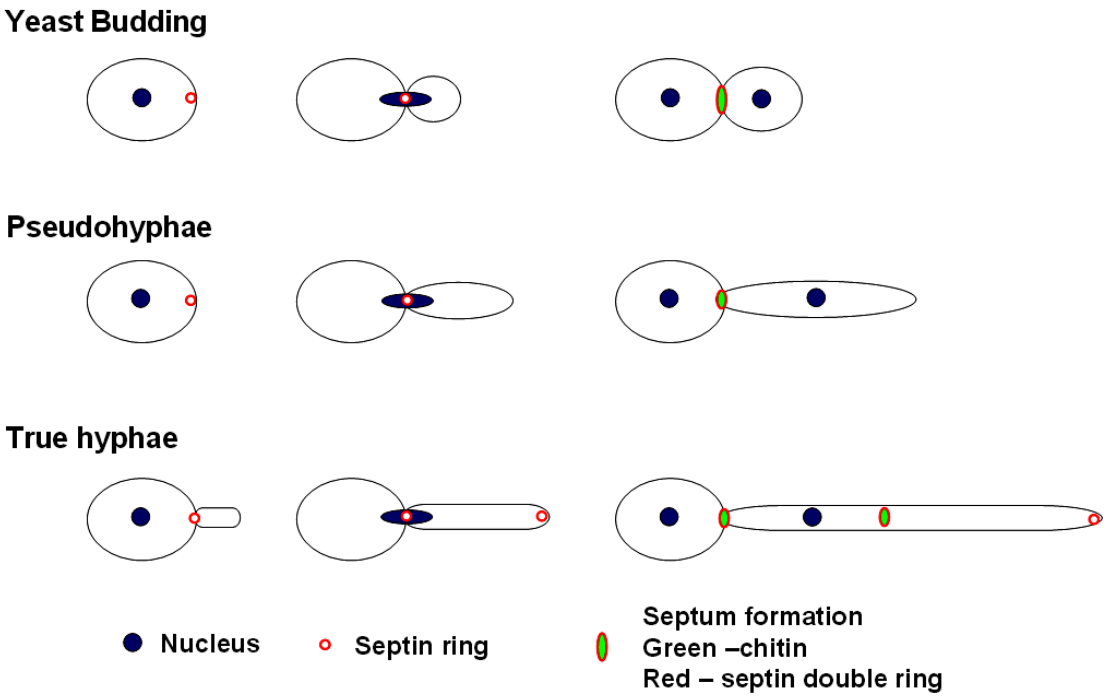
It is important to note that filamentous growth in *C. albicans* occurs in ways that produce either pseudohyphae or true hyphae. In true hyphae, the septins localize first at the initial germ tube, about 10 to 15 μm from the mother cells, and form a ring structure (Warenda and Konopka, 2002). The difference between septin ring of true hyphae and those of budding yeast and pseudohyphal cells is that the previous septin ring remains even after septation occurs. Interestingly, in *C. albicans*, septins also can be observed at the leading apex of the germ tube or hyphae (Warenda and Konopka, 2002; Figure 5). Thus, these observations suggest that septins also play a role in cell polarization in filamentous growth.

1. 3 Septins in *Schizosaccharomyces pombe*

Compared with *S. cerevisiae*, *S. pombe* has a unique method of cell separation—fission. Consistent with this, the septin function and assembly in the fission yeast are somewhat different from those of the budding yeast. For example, *S. pombe* septins are not localized at the early cell cycle stage and seem not to be essential for viability. In addition, septin complexes can be observed at the division site only after the actomyosin contractile ring is fully assembled (Berlin *et al.*, 2003; An *et al.*, 2004).

Figure 5. *C. albicans* septin localization in budding yeast, pesudohyphal, and true hyphal cells. Representations of localizations of the septin complex and other micro-organelles in each morphotype during cell cycle progression (modified from Sudbery *et al*., 2004). The septins localize first at the emerging germ tube about 10–15 μm from the mother cells and then localize at the septum as true hyphae grows. Septins also can be observed at the leading edge of the growth of the germ tube or hyphae.

Figure 5


Yeast Budding
Pseudohyphae
True hyphae
Nucleus
Septin ring
Septum formation
Green –chitin
Red – septin double ring

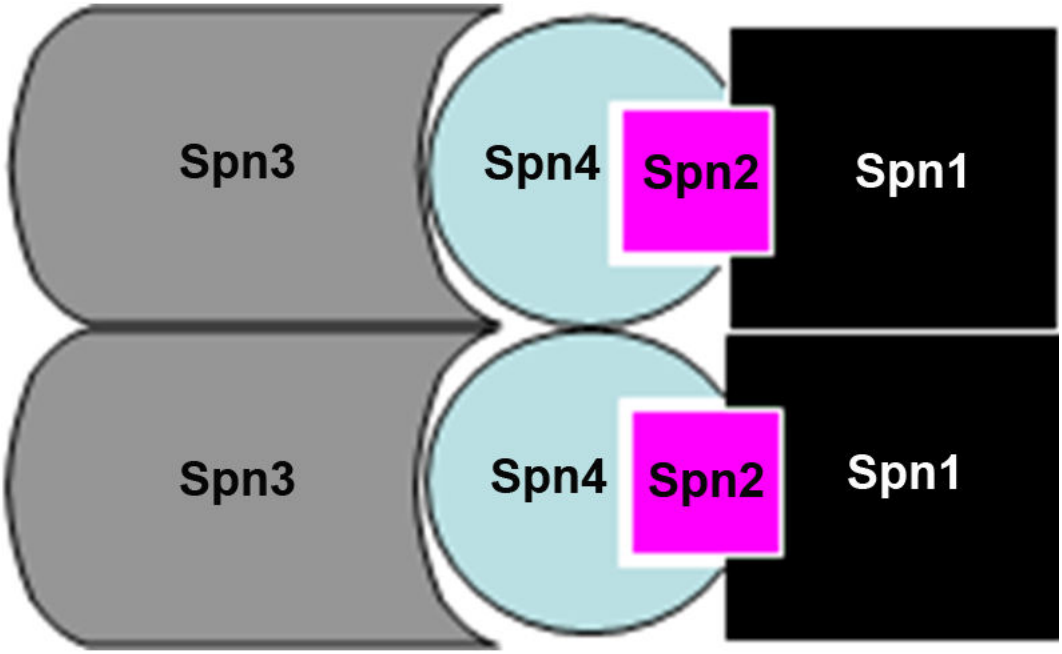
So far, through genome-wide sequencing, seven septin homologs also have been identified in *S. pombe*. Four of these—Spn1p (a Cdc3p homolog), Spn2p (a Cdc10p homolog), Spn3p (a Cdc11p homolog), and Spn4p (a Cdc12p homolog)—function during vegetative growth. Although the vegetative septin genes are not all essential for viability, lack of any or all septin genes cause delays in cell cycle progression and cytokinesis, producing a chained cell phenotype (Berlin *et al*., 2003). For the association of septin complexes in *S. pombe*, septins bind each other in a cooperative manner and in the linear order Spn3p→Spn4p→Spn1p→Spn2p (Figure 6). However, in contrast to the situation in *S. cerevisiae* (Figure 2), no *S. pombe* septin serves as a bridge protein (*i.e*. Cdc10p) for septin complex formation (An *et al*., 2004). Moreover, Mid2p, which is similar to *S. cerevisiae* Bud4p in sequence, is required for maintaining septin complex stability and integrity at cell separation (Berlin *et al*., 2003). The remaining Spn5p, Spn6p, and Spn7p septins function in the sporulation process (Mata *et al*., 2002), but the exact functions of these septins are not clear.

1. 4 Septins in *Aspergillus nidulans*.

To date, only five septins have been identified in *Aspergillus nidulans*: *aspA* and *aspE* (*CDC11* homolog), *aspB* (a *CDC3* homolog), *aspC* (a *CDC12* homolog), and *aspD* (a *CDC10* homolog) (Momany *et al*., 2001). In this filamentous, conidiogenous fungus, *aspB* is the only septin gene known to be essential for viability (Momany and Hammer, 1997): its ts mutant shows critical defects in septa and hyphal

Figure 6. *S. pombe* septins interact in a cooperative manner. The septins of this yeast associate in a linear order: Spn3p binds to Spn4p, which recruits Spn2p. Finally, Spn1p is recruited to the septin complex through Spn2p (modified from An *et al*., 2004).

Figure 6



branch formation and conidiophore (asexual spore) development (Westfall and Momany, 2002). However, the functions of the remaining septins and the interactions between the septins and other proteins are poorly understood.

1. 5 Septins in animals

In the worm *Caenorhabditis elegans*, there are two septin homologs, UNC-59 (a Cdc12p homolog) and UNC-61 (a Cdc3p homolog) which localize to the leading edge of the cell cleavage furrow and the spindle midbody. Mutagenesis studies with siRNA injection have suggested that the absence of UNC-59 and/or UNC-61 causes defects in embryonic cytokinesis and morphogenesis, germ cell development, coordination of movement, and male sensory neurons (Nguyen *et al*., 2000)

Five septins have also been identified in *Drosophila melanogaster*. Interestingly, however, a Cdc10p homolog appears to be absent in fruit fly: Sep2 and Sep5 are Cdc3p homologs, Sep1 and Sep4 are Cdc11p homologs, and Pnut is a Cdc12p homolog. These septins also function in cell separation or cytokinesis, as do their orthologs in *S. cerevisiae*. In early embryos, these septins localize at the front of cellularization movements and in dividing cells at cleavage furrows. Unexpectedly, however, Pnut is required for proper development of R7 photoreceptor cells (Neufeld and Rubin, 1994), suggesting that septins may have a novel function in fly.

In mammals, thirteen septin genes belonging to four groups have been identified: the *SEPT2* group (*SEPT1*, *SEPT2*, *SEPT4*, and *SEPT5*), the *SEPT3* group (*SEPT3*, *SEPT9*, and *SEPT12*), the *SEPT6* group (*SEPT6*, *SEPT8*, *SEPT10*, and *SEPT11*), and the *SEPT7* group (*SEPT7* and *SEPT13*) (reviewed by Spiliotis and

Nelson, 2005). However, more isoforms of septin proteins may exist in mammals because of the existence of multiple translation initiation sites and alternative splicing. In the manner of the *S. cerevisiae* septins, the mammalian septins also act as a scaffold for recruiting proteins. For example, SEPT9 and its isoforms co-localize and are co-purified with α-tubulin in mitotic cells (Nagata *et al*., 2003). Moreover, SEPT2 is required for proper localization of CENP-E (centromere-associated protein E), which is necessary for stable kinetochore attachment and depolymerization of spindle ends (reviewed by Spiliotis and Nelson, 2005). Also, septins in mammals co-localize and are structurally inter-dependent with F-actins and anillin, an actin-bundling protein that links between actins and septins (Kinoshita *et al*., 2002). Even though numerous reports document that septins have important functions in mammals, the functions of each septin as a monomer is unclear.

2. THE FUNGAL CELL WALL

The fungal cell wall is not only the outermost boundary between fungi and environment (or hosts), but also is an essential structure for fungal viability. Basically, the cell wall of a fungus plays a critical role in osmotic protection, vegetative or reproductive growth, transport of cellular macromolecules, and sporulation (Perfect, 1996). It is also an attractive target for antifungal drug development, because many of its components are absent in mammalian cells. Furthermore, the cell walls and septa are about one third of the dry weight of a fungus. Therefore, for producing cell walls,

fungi have to use a good amount of energy and synthetic activity, and regulate cell wall synthesis tightly during cell cycle progression (Cabib *et al*., 2001).

In general, the overall composition of fungal cell walls is similar: complexes of proteins and of polycarbohydrates, such as glucan, mannan, and chitin. By alkali solubilization, the polysaccharides of the cell walls are divided into two groups. First, an alkali soluble group that contains mannoproteins and α-(1, 3)D-glucan, serve as interstitial components linking and regulating the structural polysaccharides. Second, the insoluble group includes β-(1, 3)D-glucan and β-(1, 6)D-glucan, chitin, and chitosan, which act as structural components providing rigidity to the cell wall (reviewed by Debono and Gordee, 1994).

2. 1 Chitin and Chitin Synthase

Chitin (a β-1, 4-N-acetylglucosamine polymer) is an essential component of the cell wall of fungi. The distribution and amount of chitin in cell wall vary in different developmental stages, not only between fungi but even within the same fungus. For example, in *S. cerevisiae* chitin only accounts for 1–2% of the dry weight of the cell wall and is mostly found in the yeast cell bud scar and in the bud neck region at the mother and daughter cell separation site. In addition, a small amount of chitin is deposited in the yeast cell lateral wall, or in the tip of the "shmoos" formed during spore germination and under pheromone stimulation (Molano *et al*., 1980; Cabib *et al*., 1983). However, in filamentous fungi, chitin accounts for as much as 40% of the dry cell wall weight (Cabib *et al*., 1983) and localizes mostly in the septal

region and the tip of growing hyphae (Gooday, 1990), which suggests that in hyphae chitin plays a role in fungal growth.

Chitin synthases, [UDP-N-acetyl-D-glucosamine: chitin 4-β-N-acetylglucosamine transferases (EC 2. 4. 1. 16)] are responsible for chitin synthesis and for the deposition of chitin into the cell wall. After first being described in *Neurospora crassa* (Glazer and Brown) in 1957, the cloning of the first chitin synthase gene in *S. cerevisiae* (Cabib, 1987) was not achieved until 30 years later. Since then, numerous chitin synthase genes have been identified and characterized in many fungi. All chitin synthases are believed to contain membrane-bound proteins and to perform in the same way—adding a monomer of UDP-N-acetylglucosamine to the elongating polysaccharide chitin polymer (Cabib *et al*., 1983).

2. 1. 1 Chitin synthases in *S. cerevisiae*

In *S. cerevisiae*, three chitin synthase structural genes (*CHS*), encoding isozymes of three classes, are responsible for production of chitin. *CHS1* encodes a class I chitin synthase (Chs1p) that counteracts the hydrolysis activity of chitinase, which aids in yeast cell separations. In a *chs1* strain, lysis of daughter cells can occur in favor of the high activity of chitinase. Disruption of *CHS1* in *S. cerevisiae* produces no lethality and only a defect in chitin synthesis (Bulawa *et al*., 1986). The class II chitin synthase, encoded as *CHS2*, is responsible for production of the chitin that constitutes the primary septum. During cell division, the primary septum develops in the gap formed as a consequence of the septin ring and cytoplasmic membrane dividing the cells cytoplasm in two; the gap is then filled by the action of Chs2p

(Figure 7; reviewed by Cabib *et al*., 2001). This scenario is supported by a study with Myo1p (type II myosin, an essential factor of the actomyosin contractile ring). For example, in *myo1chs2* double mutants, even though some cells still maintain abnormal cytokinesis and septation, cells fail to deposit the primary septum (Dobbelaere and Barral, 2004). Instead, these mutants form a very thick and amorphous septum (with no thin chitin line visible by transmission electron microscopy) that has no chitin for chitinase to act upon. The end result is defects occur in that cell separation and large cell aggregates are formed. Furthermore, even though they localize aberrantly, Myo1p, Chs2p, and Cdc12p are co-localized in a *cdc3* mutant background (Roh *et al*., 2002). This indicates that Chs2p possibly interacts with a septin (*i.e.* Cdc12p) and thereby localizes at the bud neck. However, the exact mechanisms that bring about Chs2p activation (Chsp isozymes, at least *in vitro*, are all zymogenic) and localization are not yet clear. Finally, the *CHS3* gene encodes the catalytic sub-unit of the class IV chitin synthase Chs3p, which is responsible for chitin ring formation and which produces the majority of cell wall chitin (Figure 7; reviewed by Cabib *et al*., 2001). At the time of bud emergence, Chs3p localizes at the bud neck through an interaction with Chs4p (a Chs3p activation regulator), which in turn associates with Bni4p (a bud neck scaffold protein) that is then finally recruited by Cdc10p to the bud neck (De Marini *et al*., 1997; Figure 8). Taken together these findings indicate that septins are important for recruitment of chitin synthase to the bud neck, thereby allowing for the deposition of chitin in the bud neck and its participation in cytokinesis.

Figure 7. Cell wall growth and septum formation. The cell wall is in depicted gray, and the plasma membrane is represented by the red broken line. (A) Polarized yeast bud emergences, Chs3p (green) deposits chitin to form the chitin ring (grey). (B) As the bud enlarges, isotropic growth starts. When the chitin ring invaginates at cytokinesis, Chs2p (black) synthesizes the primary septum chitin (grey) concomitantly. (C) When the primary septum is completed, secondary septal chitin (yellow) is deposited on each side by the mother and daughter cells, respectively. With the aid of chitinase digestion, mother and daughter cells are asymmetrically separated. Chs1p counteracts chitinase. BS: bud scar (modified from Cabib *et al*., 2001).

Figure 7

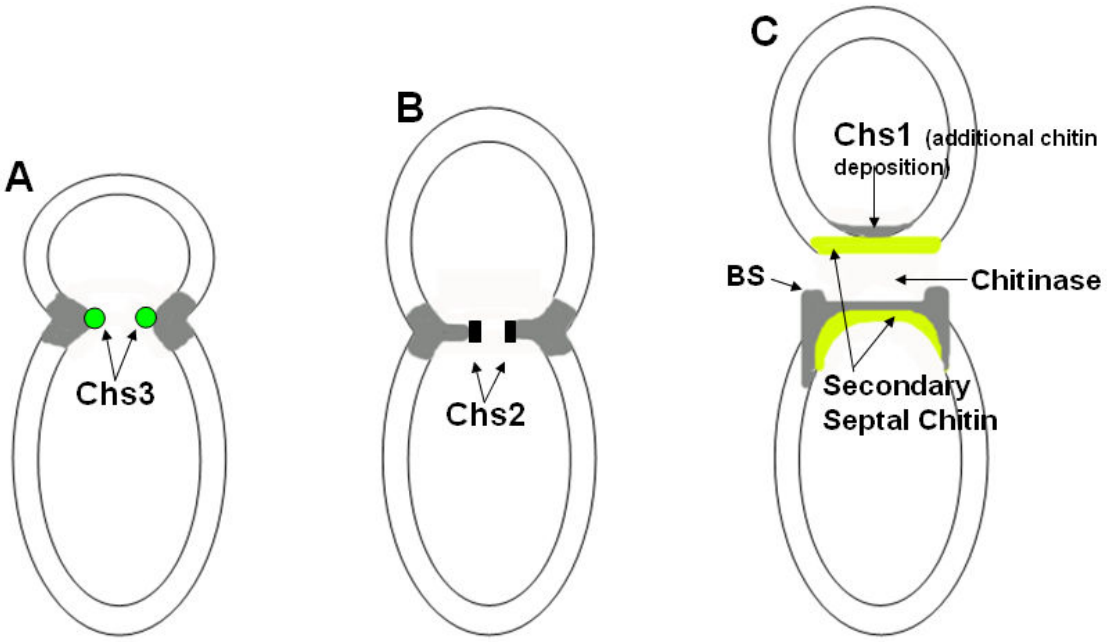
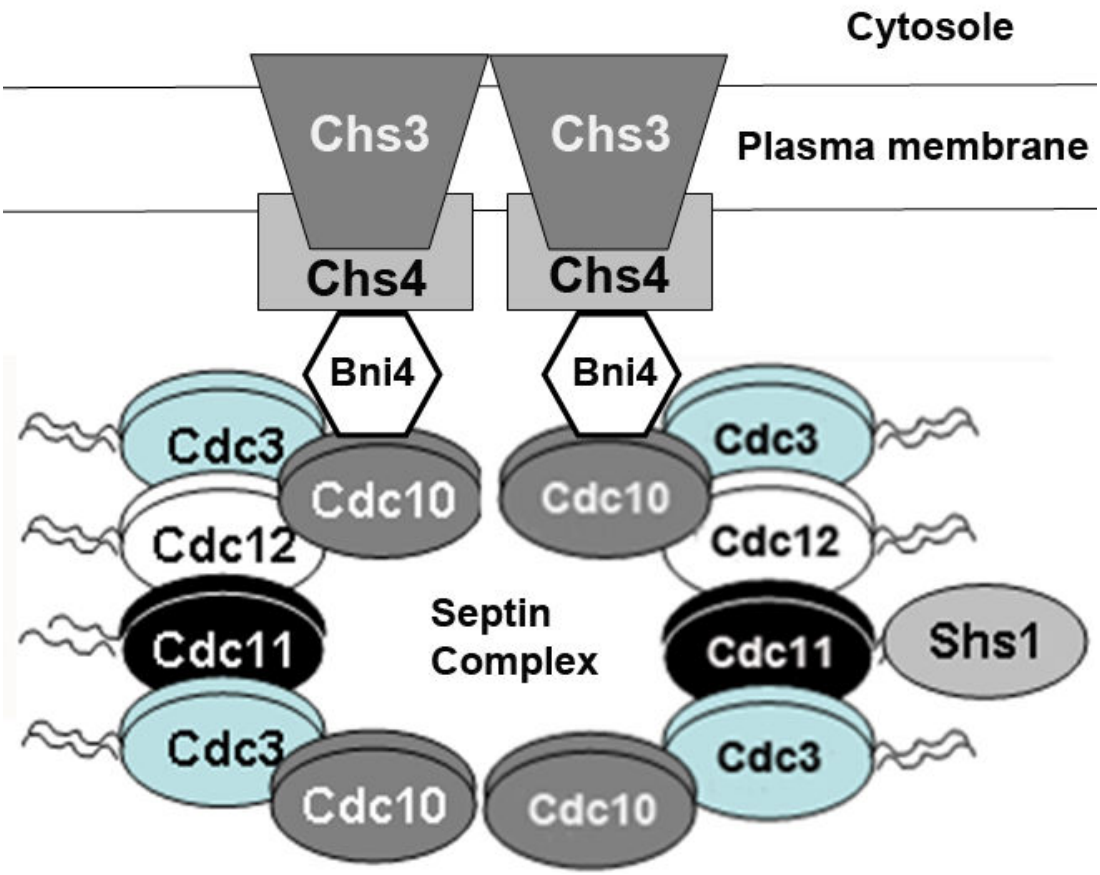

A
Chs3
B
Chs2
C
Chs1 (additional chitin deposition)
BS
Chitinase
Secondary Septal Chitin

Figure 8. The interactions among the chitin synthase III complex, Bni4p, and the septins. Chs3p localizes at the bud neck through an interaction with Chs4p, which in turns associates with Bni4p, which is then finally recruited by Cdc10p to the bud neck (modified from De Marini *et al*., 1997).

.

Figure 8


Cytosole
Chs3
Chs3
Plasma membrane
Chs4
Chs4
Bni4
Bni4
Cdc3
Cdc10
Cdc10
Cdc3
Cdc12
Cdc12
Septin
Complex
Cdc11
Cdc11
Shs1
Cdc3
Cdc10
Cdc10
Cdc3

2. 1. 2 Chitin synthases in fungi able to grow filamentously

Among filamentous fungi, the chitin synthases of *C. albicans*, *A. fumigatus*, and *A. nidulans* have received considerable attention. First, the medically important dimorphic fungus, *C. albicans*, has three chitin synthases structural genes. The *C. albicans* Chs1p (class II) is found to be essential for viability. Moreover, it is also required for septum formation and the maintenance of the lateral cell wall integrity (Munro *et al*., 2001). Chs2p (class I) in *C. albicans* is not required for viability, but contributes about 80% of the total chitin synthase activity *in vitro* (Munro *et al*., 1998). Finally, *C. albicans chs3* mutants show 80–90% reduction in the cell wall chitin content, but exhibit normal growth and cell shape (Bulawa *et al*., 1995). The recruitment of chitin synthase to the bud neck in this fungus is poorly understood. Second, in the medically important *A. fumigatus*, seven chitin synthase genes have been cloned and characterized, but none found to be essential. These encode proteins belonging to five classes: *A. fumigatus* ChsA (class II), *A. fumigatus* ChsB (class II), *A. fumigatus* ChsC and ChsG (class III), *A. fumigatus* ChsF (class IV), *A. fumigatus* ChsE (class V), and *A. fumigatus* ChsD (class VI) (Munro *et al*., 2001). When *A. fumigatus CHSA*, *CHSB*, *CHSD*, and *CHSF* are mutated individually, the cells of each single chitin synthase mutant show normal growth and morphology (Medallo *et al*., 1995). However, *A. fumigatus chsC*, *chsE*, and *chsG* mutants show reduced chitin content, abnormal hyphal morphology, poor growth, and defects in conidiogenesis (Aufauvre-Brown *et al*., 1997). To date, the interactions between the septins and chitin synthases and their localization in *A. fumigatus* remain unclear. Third, unlike the pathogenic mold *A. fumigatus*, the nonpathogenic *Aspergillus* species, *A. nidulans*, has

six chitin synthase genes, *CHSA*, *CHSB*, *CHSC*, *CHSD*, *CSMA*, and *CSMB*, which encode isozymes of classes II, III, I, IV, V, and VI, respectively. Of these, only ChsB is an essential for normal hyphal growth (Yanai *et al.*, 1994). Even though single mutants of *chsA*, *chsC*, or *chsD* did not show significant defects in growth and morphology compared with WT, some double mutants (*i.e. chsAchsC*) have been shown to be functionally overlapped in asexual reproduction, septum formation, and hyphal development (Motoyama *et al*., 1996; Fujiwara et al., 2000; Ichinomiya *et al*., 2002). CsmA, a class V chitin synthase, consists of a C-terminal catalytic domain and an N-terminal myosin motor-like domain. Its mutant showed defects in lateral cell wall integrity, especially under low osmotic pressure, and hyphal morphology (Horiuchi *et al*., 1999). Moreover, CsmA binds to actin structures near the hyphal tip and in the septal region. This interaction between CsmA and actin is required for not only the proper localization of CsmA but also its function (Takeshita *et al*., 2005). Recently, a class VI chitin synthase, CsmB, has been characterized, and it has been determined that it also has a myosin motor-like domain and overlapping functions in hyphal development in *A. nidulans* (Takeshita *et al*., 2006). However, again, the interactions between septins and chitin synthases in *A. nidulans* are still poorly understood.

3. *Wangiella dermatitidis*: classification and biology

Wangiella dermatitidis is a melanized asexual fungus and a well-known causative agent of the human disease phaeohyphomycosis. First reported on and

named by Kano (Kano, 1934) as *Hormiscium dermatitidis*, it has since been reported as a number of different species including *Exophiala dermatitidis* and *Wangiella dermatitidis* (McGinnis, 1977), based upon various observations. Even though it is confirmed by a number of molecular phylogenetic studies (De Hoog *et al*., 1994) that *E. dermatitidis* and *W. dermatitidis* refer to the same species, to keep consistency with the previous studies of this fungus performed by the Szaniszlo group, *W. dermatitidis* is applied to the species used in my research. Because *W. dermatitidis* is asexual, it can not be classified morphologically. However, it is molecularly to be classified among certain ascostromatic ascomycetes (Figure 9).

3. 1 Polymorphic transitions in *W. dermatitidis*

The conidiogenous mold, *W. dermatitidis* is polymorphic and has a number of vegetative growth forms (morphotypes), including budding yeast, pseudohyphae, moniliform hyphae, true hyphae, isotropically enlarged (non- septated) yeast cells, and sclerotic (multi-cellular) bodies (reviewed by Szaniszlo, 2006). It grows predominantly as a budding yeast form, particularly in rich nutrient environments, but it transitions easily to other growth forms in environments with fewer nutrients or under other stress conditions (Figure 10). *W. dermatitidis* yeast daughter cells are derived from their yeast parents, hyphal blastospores, or conidia (Oujezdsky *et al*., 1973). The conversion of yeast cells to hyphae is achieved under conditions such as prolonged culture, transient acidity, or nitrogen source limitation, which induce yeast cells first to form so-called moniliform (pseudohyphae-like) hyphae, and then

Figure 9. Molecular taxonomic classification of *W. dermatitidis*.

Figure 9

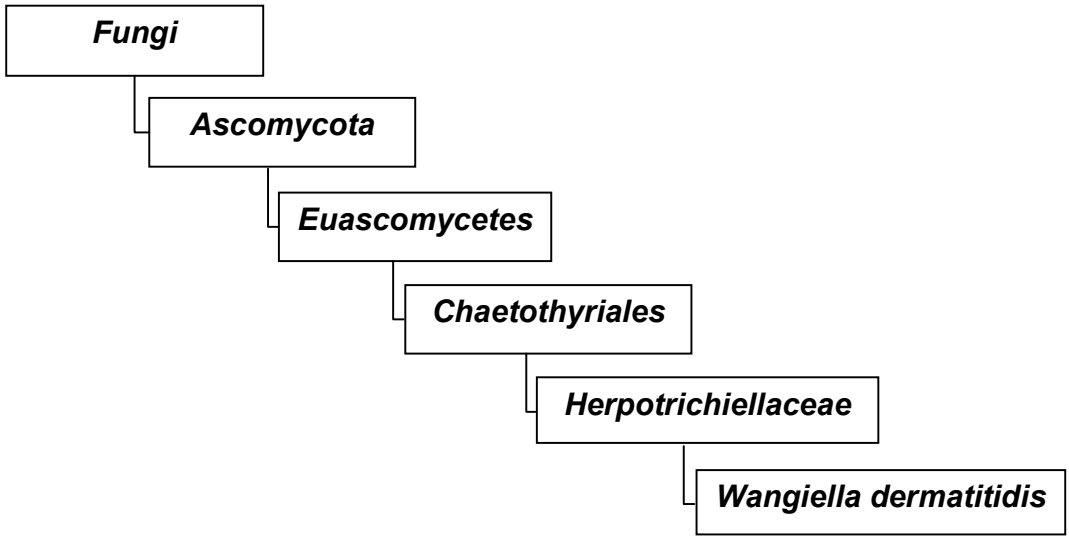
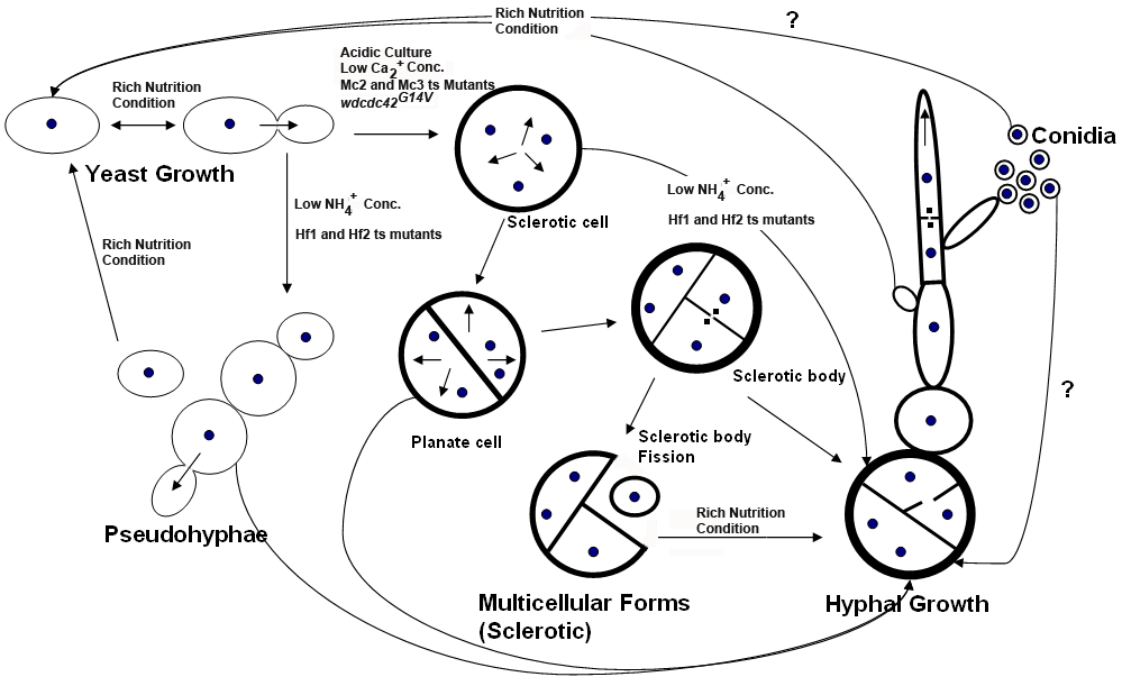

Fungi
Ascomycota
Euascomycetes
Chaetothyriales
Herpotrichiellaceae
Wangiella dermatitidis

Figure 10. *W. dermatitidis* polymorphogenic development. Solid arrows indicate the changes in cell polarity during morphogenic processes. Black indicates cell walls and septa and grey circles represent nuclei (modified from Szaniszlo, 2006).

Figure 10


Rich Nutrition Condition
?
Acidic Culture
Low Ca2+ Conc.
Mc2 and Mc3 ts Mutants
wdcdc42G14V
Rich Nutrition Condition
Yeast Growth
Low NH4+ Conc.
Hf1 and Hf2 ts mutants
Sclerotic cell
Low NH4+ Conc.
Hf1 and Hf2 ts mutants
Conidia
Rich Nutrition Condition
Sclerotic body
?
Planate cell
Sclerotic body Fission
Rich Nutrition Condition
Pseudohyphae
Multicellular Forms (Sclerotic)
Hyphal Growth

eventually true hyphae (Figure 10). Also, certain mutants such as the temperature-sensitive mutant, Hf1, produces hyphae from yeast by shift from 26°C to 37°C (McIntosh, 1996). The morphological transition of yeast to the sclerotic morphotypes is induced by prolonged culture in acidic media of pH 2.5 or by calcium limitation at pH 6.5. Again certain temperature-sensitive mutants, such as Mc1, Mc2, and Mc3 can be induced to form multi-cellular sclerotic bodies at the restrictive temperature of 37 °C (Roberts and Szaniszlo, 1978). Studies have shown that the cells of the sclerotic-induced morphotypes, including those of the mutants, show normal macromolecule synthesis at the high temperature, suggesting that multi-cellularity is acquired by inhibition of bud emergence and cell separation without prohibition of normal nuclear division and growth (Roberts and Szaniszlo, 1978). The hyphal and sclerotic body growth forms induced from the *W. dermatitidis* yeast cells are thought to be stress-response forms that possibly enhance the virulence of this pathogen.

W. dermatitidis has many attractive merits as a research model: (1) the yeast phase of this conidiogenous; but polymorphic mold allows exact quantification, which is hard with obligatory filamentous species; (2) molecular biology-based transformation and genetic manipulation are available; (3) parasexually derived diploid strains exist, which allows the examination of the essentiality of genes (Cooper *et al*., 1993); and (4) a variety and a number of cloned and characterized genes have been proven to encode products related to viability, virulence, and cellular development and differentiation (reviewed by Szaniszlo, 2002; Szaniszlo, 2006). Thus, *W. dermatitidis* is a particularly good model system for my studies of fungal cellular development and differentiation, because it has the potential to reveal

considerably more information about the importance of septins to growth in yeast and hyphae, and possibly even in the unique sclerotic morphotypes associated with this pathogen (reviewed in Szaniszlo, 2002; Szaniszlo, 2006).

3. 2 Cell wall composition of *W. dermatitidis*

It has been proposed that the cell wall of the black yeasts including *W. dermatitidis* plays an important role in their pathogenicity. In *W. dermatitidis*, chitin and 1, 8-dihydroxylnaphtalene (DHN) melanin, two major cell wall components have received considerable attention (reviewed by Szaniszlo, 2002; Szaniszlo, 2006). The cell wall melanin deposition increased at high temperature or during yeast to sclerotic body transition (Szaniszlo *et al*., 1983; Copper *et al*. 1984). In a mouse model system, non-melanized *W. dermatitidis* strains such as *wdpks1* (Feng *et al*., 2001) showed less virulence than melanized strains (Dixon *et al*., 1992; Feng *et al*., 2001). Melanin is believed to be a virulence factor because it provides UV or oxidation resistance to fungal cell (Jacobson *et al*., 1995) and allows fungi to penetrate their plant hosts (Chumley and Valent, 1990). Moreover, WT *W. dermatitidis* cells grown in a low-pH medium become less melanized and show less resistance to zymolyase, a 1,3-β-glucanase treatment, while melanized wild type cells are resistant to zymolyase (Montijn *et al*., 1997). Furthermore, the cells of the *mel3*, an albino mutant strain, show even more sensitivity to zymolyase and to low pH (Montijn *et al*., 1997), suggesting that melanin also provides osmotic and zymolyase resistance to the *W. dermatitidis* cell wall.

Chitin is an important cell wall component of *W. dermatitidis*. It is deposited mainly in septal regions, including mother and daughter cell junctions and bud and birth scars of yeast, and also in the side walls of hyphae and sclerotic forms. Among the morphotypes, the most chitin is probably deposited in the inner wall layers of sclerotic body cells (Harris and Szaniszlo, 1986).

3. 3 Chitin synthase in *W. dermatitidis*

To date, five chitin synthase structural genes (*WdCHS*) of *W. dermatitidis* have been identified, cloned, and each found to encode a chitin synthase of a different class by the Szaniszlo group. WdChs1p, a class II chitin synthase and orthologue of *S. cerevisiae* chitin synthase II, is thought to be responsible for production of the primary septum prior to yeast cell separation: *wdchs1*Δ mutant cells fail to separate efficiently and instead form short yeast chains, like pseudohyphae (Zheng *et al*., 2006). This implies that WdChs1p could have a relationship with septins in a manner similar to that of Chs2p in *S. cerevisiae*. WdChs2p is a class I chitin synthase. In *wdchs2*Δ mutants, only negligible morphological changes occur, but dramatic reductions in chitin synthase activity are evident at both 26 ºC and 37 ºC. No loss of virulence with these two mutants is observed in a mouse model system (Wang *et al*., 2001). Therefore, WdChs2p also appears to act as a repair enzyme, although its function may be redundant with that of WdChs1p (Zheng *et al*., 2006), which is consistent with the situation with *S. cerevisiae* Chs1p (Bulawa *et al*., 1986). WdChs3p (class III) does not have an *S. cerevisiae* homologue (Wang and Szaniszlo, 2000). Although mutant cells without WdChs3p show normal growth and morphology compared with the WT cells

(Wang and Szaniszlo, 2000), when shifted to 37°C the WdChs3p expression level increases dramatically, suggesting that WdChs3p plays an important role under stress conditions (Wang and Szaniszlo. 2000). Surprisingly, even though single *wdchs2* and *wdchs3* mutants do not have reduced virulence, the disruption of both *WdCHS2* and *WdCHS3* in the same background results in mutants with reduced virulence in a mouse model system even though they also show normal growth (Wang *et al*., 2001). It is suggested that WdChs2p and WdChs3p compensate for each other at the temperature and environment of infection required for *W. dermatitidis* survival in mice. However, the basis of loss of virulence of this double mutant strain is unknown. The class IV chitin synthase of *W. dermatitidis*—WdChs4p—synthesizes a large amount of chitin and appears to play a role in cytokinesis because *wdchs4*Δ mutant strains have an abnormal yeast phenotype, such as tending to clump, being hyper-pigmented, and having multiple buds (Wang *et al*., 1999). This strongly suggests that WdChs4p, in the manner of its *S. cerevisiae* homolog Chs3p, has a role in cell separation and may directly associate with septins and thereby act in cytokinesis. Finally, the class V chitin synthase, WdChs5p of *W. dermatitidis* also has no *S. cerevisiae* homologue (Liu *et al*., 2004). Importantly, WdChs5p is the only single-chitin synthase required for *W. dermatitidis* viability, at least at 37 ºC (Liu *et al*., 2004). The *wdchs5* mutant strains show defects in growth, cell wall integrity, and chitin distribution at high temperature, but only after relatively prolonged culture (Liu *et al*., 2004). Moreover, the *wdchs5*Δ*-11*, cells are frequently clumped, chained, and enlarged only after relatively prolonged culture at 37°C (Liu *et al*., 2004), indicating that WdChs5p may have both a role in cell separation and an interaction with *W.*

dermatitidis septins. Consistent with the *A. nidulans* homolog, CsmA (class V, Takeshita *et al*., 2006), WdChs5p also has a C-terminal chitin synthase domain and an N-terminal myosin motor-like domain (Liu *et al*., 2004), indicating that, like CsmA, it may localize too at the tips of hyphae or in septal regions (Takeshita *et al*., 2006). However, in *W. dermatitidis* the exact cellular or molecular functions of all of the chitin synthases and their biological interactions and localizations with other important proteins (*i.e.* septins) are completely unknown, except that all appear to be membrane-bound enzymes. Because, it is clear that the reduction of virulence in *W. dermatitidis* is sometimes correlated with loss of chitin synthase function (reviewed Szaniszlo, 2002; Szaniszlo, 2006), the goal of my research was to increase our understanding of chitin synthesis and localization in *W. dermatitidis* by investigating the functional roles of septins and their interactions with the chitin synthases during cellular development and differentiation.

II. MATERIALS AND METHODS

A. Strains

The strains used in this study are listed in Table 1. The wild type (WT) *W. dermatitidis* (strain 8656; ATCC 34100); a parasexually derived diploid 3u2m-428 (*mcm2*/*cdc1 mcm3*/*cdc2 met ura mel3 mel4*); and the chitin synthase mutants *wdchs1*Δ*-1*, *wdchs2*Δ*-1*, *wdchs3*Δ*-1*, *wdchs4*Δ*-1*, and *wdchs5*Δ*-11* were obtained from Dr. Paul J. Szaniszlo (University of Texas at Austin). The *Saccharomyces cerevisiae* ts mutant strains M1621 (*MATa, cdc3-6, his3, leu2, lys2, trp1, ura3*), M1620 (*MATa, cdc10-1, his3, leu2, lys2, trp1, ura3*), M1723 (*MATa, cdc11-6, his3, leu2, lys2, trp1, ura3*), and M1726 (*MATa, cdc12-6, his3, leu2, lys2, trp1, ura3*) were kindly provided by Dr. John R. Pringle (University of North Carolina, Chapel Hill). The strain XL1-Blue (Strategene, La Jolla, CA) of *Escherichia coli* was used for the amplification of sub-genomic libraries, cloning, and plasmid preparation.

B. Growth media and conditions

The synthetic minimal medium SD and the rich medium YPD were used for preparation of *Wangiella dermatitidis* cells. The composition (W/V) of SD broth was prepared as follows: 1% dextrose, 0.17% Difco^{TM} yeast nitrogen base without amino acids and ammonium sulfate (BD, Sparks, MD), 0.2% ammonium nitrate, and 0.1% asparagines (Sigma, USA). The pH was adjusted to 6.5.

Table 1. Strains used in this study.

Strain	Genotype	Reference or Source
Wangiella dermatitidis		
Wd8656	Wild type (WT)	ATCC34100
3u2m-428	Diploid WT	Cooper and Szaniszlo, 1993
*wdchs1*Δ*-1A*	*wdchs1::hph*$^{+}$	Li *et al*., 2006
*wdchs2*Δ*-1*	*wdchs2::hph*	Wang *et al*., 2001
*wdchs3*Δ*-1*	*wdchs3::hph*	Wang and Szaniszlo, 2000
*wdchs4*Δ*-1*	*wdchs4::hph*	Wang *et al*., 1999
*wdchs5*Δ*-11*	*wdchs5::hph*	Liu *et al*., 2004
*wdcdc3*Δ*-1*	*wdcdc3::hph*	This study
*wdcdc3*Δ*-2*	*wdcdc3::sur*$^{++}$	This study
*wdcdc10*Δ*-1*	*wdcdc10:sur*	This study
*wdcdc11*Δ*-1*	*wdcdc11::hph*	This study
*wdcdc12*Δ*-1*	*wdcdc12::sur*	This study
*wdcdc10Δ-1wdchs2*Δ*-1*	*wdchs2::hph wdcdc10:sur*	This study
*wdcdc10*Δ*-1wdchs3*Δ*-1*	*wdchs3::hph wdcdc10:sur*	This study
*wdcdc10*Δ*-1wdchs4*Δ*-1*	*wdchs4::hph wdcdc10:sur*	This study
*wdcdc10*Δ*-1wdchs5*Δ*-11*	*wdchs5::hph wdcdc10:sur*	This study
Saccharomyces cerevisiae		
CWY1	*MAT a his3 leu2 lys2 trp1 ura3*	Lab stock
M1621	*MAT a his3 leu2 lys2 trp1 ura3 cdc3-6*	See text
M1620	*MAT a his3 leu2 lys2 trp1 ura3 cdc10-1*	See text
M1723	*MAT a his3 leu2 lys2 trp1 ura3 cdc11-6*	See text

Table 1, cont.

M1726	*MAT a his3 leu2 lys2 trp1 ura3 cdc12-6*	See text

$^{+}$*hph*: hygromycin resistance gene. $^{++}$*sur*: sulfonylurea resistance gene.

After autoclaving, dextrose autoclaved separately was added into the medium. The YPD broth contained 2% dextrose, 2% peptone, and 1% yeast extract (Sigma). Drug selection plates and broth for isolating *W. dermatitidis* transformants were made by adding hygromycin B (HmB, Sigma) to YPD at a final concentration of 20 μg/ml for resistance conferred by the hygromycin B phosphotranferase (*hph*) gene and chlorimuron ethyl (Chem Service, West Chester, PA) was added to SD at a final concentration of 20 μg/ml for resistance conferred by the sulfonylurea resistance (*sur*) gene. For inducing the morphological transitions to hyphae and conidiogenesis of *W. dermatitidis*, cells were cultured on potato dextrose agar (PDA) for 20 days at 26°C. The composition (W/V) of PDA plates was prepared as follows: 20% potato infusion (Difco, Detroit, MI), 2% dextrose, and 1.5% agar (Sigma). For temperature-induced morphological transitions, early log-phase yeast cells (10^6 cells/ml) of the *W. dermatitidis* strains were cultured in YPD broth at 26 °C, and then transferred to pre-warmed YPD broth and grown for an additional 24 h at 37 °C. The *E. coli* XL-1 Blue strain was first cultured at 37 °C for one day with Luria-Bertani (LB) plates or broth, supplemented with ampicillin (100 μg/ml, Sigma) or chloroamphenicol (25 μg/ml, Sigma).

C. Enzymes, molecular weight marker, and molecular biology kits

Restriction enzymes were purchased from New England BioLabs (NEB, Beverly, MA) and Promega (Madison, WI). The T4 DNA ligase was purchased from Invitrogen (USA). Taq polymerases were purchased from Invitrogen (USA) and Takara (Japan). DNase (RNase free) and RNase (DNase free) were purchased

from Roche (Indianapolis, IN). All of these enzymes were used according to the manufacturers' instructions and with the provided buffer. Zymolase was purchased from Zymo Research (Orange, CA), and the DNA molecular weight markers were from NEB.

For cloning PCR products, the pGEM®-T EASY vector system was used. The QIAprep spin Miniplasmid kit (QIAGEN, Chatsworth, CA) and Miniprep Express Matrix (Qbiogen, Morgan Irvine, CA) were used to isolate plasmid DNA. To recover DNA from the agarose gels, the QIAquick gel extraction kit (QIAGEN) was used. The QIAGEN One-Step RT-PCR Kit® was from QIAGEN. The DECA prime II DNA labeling kit was purchased from Ambion (Austin, TX). A Sephadex G-50 column (Böehringer MannHeim Biochemicals, Indianapolis, IN) was used to remove unincorporated nucleotides.

D. Radioisotopes, membranes, autoradiography, miscellaneous equipment, and photography

α-^{32}P-dATP and α-^{32}P–dCTP (Ci/mM) were purchased from Amersham Life Science (Arlington Heights, IL) and were used for radioisotope labeling.

Nytran nylon transfer membranes and Nytran super-charged membranes (Schleicher & Schuell, Keene, NH) were used for RNA and DNA blots, such as Southern and Northern blotting, plaque lifts, and colony hybridizations.

X-OMAT film (Kodak) and X-ray film (Research Products International Corp., Chicago, IL) were used for Southern and Northern blotting. The hybridizations of Southern blots were performed in PersonalHyb hybridizer (Stratagene, La Jolla, CA).

Electroporation was performed with a Bio-Rad Gene Pulser (BioRad Lab, Hercules, CA). PCR reactions were performed with Perkin Elmer Thermocyclers (Norwalk, CT). For DNA imaging, a Fotoprep I system trans-illuminator and camera (Fotodyne, New Berlin, WI) with Polaroid Type 667 instant film (Polaroid, Cambridge, MA) were used.

E. Nucleic acid manipulations

The genomic DNA of *W. dermatitidis* was prepared as previously described (Liu *et al*., 2004) using a DNase-free glass bead method modified from Charles S. Huffman (Ausubel *et al*., 1989). Breifly, about 1 x 10^8 cells were washed with dH_2O by centrifugation. Breaking buffer (200 µl, 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0), 200 µl glass beads (400–520 µm diameter, Thomas Scientific, Swedesboro, NJ), and 200 µl phenol/chloroform/iso-amyl alcohol (25:24:1) were added and vortexed vigorously in a Multi-tube Vortexer (VWR, West Chester, PA) for 5 min then the mixture was centrifuged at top speed for 6 min. The surface layer was carefully transferred into a fresh tube containing 1 ml of ice-cold 100% isopropyl alcohol. After a short mixing, this mixture was centrifuged again at top speed for 6 min. After carefully draining liquid, the isolated genomic DNA (pellet) was washed with ice-cold 70% ethanol and treated with RNase (DNase free, Roche) to eliminate RNA contamination. For RNA isolation, about 10^8 cells were harvested and washed with distilled water, and then the cells were treated with zymolyase to obtain spheroplasts. Finally, total RNA was prepped by extraction with TRizol® LS reagent (Invitrogen)/Chloroform (5:1). Isolated RNA was treated with

RNase-free DNase (Rosche) at 37 °C for 1 h, followed by phenol/chloroform/iso-amyl alcohol (25:24:1) extraction and ethanol precipitation, as described previously (Liu *et al*., 2004). The DNA free-RNA pellets were dissolved in 50 µl DEPC-dH_2O.

F. Southern blot analysis

For the Southern analysis bout 4-5 µg *W. dermatitidis* genomic DNA was first digested with appropriate restriction enzymes, which was then separated by a 0.8% agarose gel electrophoresis, denaturated with 0.25 M HCl and neutralized with a 0.5-M NaOH/1.5-M NaCl solution, prior to transfer to a Nytran membrane by capillary blotting in 20X SSC buffer (3 M NaCl and 0.3 M sodium citrate). After the DNA was cross-linked to the Nytran membranes in a UVP CL-1000 ultraviolet cross-linker for 3 min, the DNA-bound membranes were first pre-hybridized in the aqueous pre-hybridization buffer (APH): 5X SSC, 0.1% (W/V) Ficoll (Sigma), 0.1% (W/V) polyvinyl pyrolidine (Sigma), 0.1% (W/V) BSA (Sigma), and 1% SDS at 68 °C for 1 h. Then probes labeled with α-^{32}P-dATP or α-^{32}P –dCTP using the DECA prime II DNA labeling kit (Ambion) were added and hybridized overnight. After hybridization, the membranes were then washed with the low-stringency wash buffer (2X SSC and 0.1% SDS) for 5 min at 68 °C, and then washed a second time with a high-stringency wash buffer (0.5X SSC and 0.1% SDS) for 5 min at 68 °C. When necessary, extremely high-stringency wash buffer (0.1X SSC and 0.1% SDS) was also used for washing. Finally, membranes were exposed to the X-ray film at -70°C for 3 to 24 h.

G. Primers and PCR amplification

Primers used in this study are listed in Table 2 and were synthesized by Invitrogen (Carlsbad, CA). Degenerative PCR amplifications using Taq polymerases from Invitrogen or Takara (Japan) were carried out in a Perkin Elmer Thermocycler (Norwalk, CT). The PCR reaction mixture contained 2.5 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 10 mM dNTPs, 10 pmol primers (each), 0.1 µg genomic DNA, and 1 unit Taq polymerase (Takara, Japan). The following program was used for degenerative PCR: one cycle of 4 min at 94°C for template DNA denaturation; 30 to 35 cycles of amplification with 1 min at 94°C, 1 min at 42°C for annealing (when necessary increased by 2°C), and 1 min at 72°C for extension; followed by an additional extension cycle of 10 min at 72°C.

H. RT-PCR

The RT-PCR was carried out using the QIAGEN One-Step RT-PCR Kit®. The reaction of 25 µl contained 1 µl $MgSO_4$ (25 mM), 0.5 µl dNTP (10 mM), 2.5 µl buffer (10X), 1 µl forward and reverse primers (20 pmol), 2 µl one-step enzyme mix, 2 µl of 1 µg mRNA isolated from the WT cells grown at 26°C or 37°C, and RNase-free DEPC-treated water added to 25 µl. The RT-PCR amplifications were performed with a GeneAmp 2400 PCR System (Perkin Elmer, Wellesley, MA) using the following conditions: 48°C for 1 h, 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 50°C for 1 min, and 68°C for 3 min, then an extra step of elongation at 68°C for 5 min.

I. Construction and screening of the sub-genomic libraries

The sub-genomic libraries of *W. dermatitidis* septin genes were constructed using genomic DNA of the WT strain completely digested with *Eco*RI for *WdCDC3*, *Hind*III for *WdCDC10*, *Eco*RI for *WdCDC11*, and *Hind*III for *WdCDC12*. After 1.2% agarose gel electrophoresis, the resulting 3-Kb (*WdCDC3*), 5-Kb (*WdCDC10*), 3.2-Kb (*WdCDC11*), and 7-Kb (*WdCDC12*) fragments were recovered and ligated into the appropriate vectors (Table 3). The resulting plasmids were cut by *Eco*RI or *Hind*III and dephosphorylated with calf intestine alkaline phosphates (CIPA, Promega, Madison, WI). The ligation mixture was then transformed into the *E. coli* competent cells. The resulting subgenomic libraries were screened by colony hybridization using the degenerative PCR products of the septin gene fragments as probes. The screening was performed as described previously (Feng *et al*., 2001). Briefly, the subgenomic libraries were first spread on LB plates containing 100 μg/ml ampicillin and incubated at 37°C overnight. Nylon membranes were placed on top of the plates for 4 min, and then the colonies bound on the membranes were lifted and transferred to 1.5 M NaCl/0.5 M NaOH solution for 5 min, 1.5 M NaCl/0.5 M Tris-HCl (pH 8.0) solution for 5 min, and 0.2 M Tris-HCl (pH 7.5)/2X SSC solution for 5 min. After cross-linking DNAs onto the membranes using a UVP CL-1000 ultraviolet crosslinker for 3 min and rinsing with 3X SSC, 0.1% SDS solution, these DNA bound membranes were used for Southern blot analyses which were fulfilled using each septin gene fragment from the degenerative PCR as a probe. The colonies of interest were isolated from the agar media

Table 2. Primers used in this study

Primers	Sequences (5' → 3')*	Characteristics
Degenerative PCR primers+		
CDC3. CF1	GGNTAYGTNGGNTTYGCNAAY	F
CDC3. CF2	GAYATHGARGARAAYGGNGTN	F
CDC3. CR1	NCCCCANGGRTANWSNCKNCC	R
CDC3. CR2	NGCNACYTTYTGYTGRAANAC	R
CDC10. C1p	CCNATHGTNAARTAYATHAAR	F
CDC10. C2p	AARGARYTNACNGCNCARMGN	R
CDC10. C3p	NCCNACNACNGCRAANGGDAT	F
CDC10, C4p	NGTNGTYTCDATNARRTCYTG	R
CDC11. C1p	AARAARYTNATHATGGARGAY	F
CDC11. C2p	RTCYTCCATDATNARYTTYTT	R
CDC11. CF1	CCNACNGGNCAYGGNYT	F
CDC11. CF2	TAYGARAAYTAYMGNAC	F
CDC11. CR1	NGTNCKRTARTTYTCRTA	R
CDC11. CR2	NCKNARYTTYTCYTCYTC	R
WdCDC12. 1p	TTGACGCGAGTGCAGAG	R
WdCDC12. 2p	TATGTGAACAATCGCGAC	R
Primers for septin gene cloning		
WdCDC3. 1p	GCGGATCC**ATG****CGAAATGTGGTCAGAAG	*Bam*HI, F
WdCDC3. 2p	CGGGATCC**TCA**TCGAAGCGAGAAGCC	*Bam*HI, R

Table 2, cont.

WdCDC3. 3p	GGATCCTAGAGGCCGGTCGTCTTTTT	*Bam*HI, F
WdCDC3. 4p	ATCGATACAGTCTATGCCGGATTTGG	*Cla*I, R
WdCDC10. 1p	GGATCCCGGAGCCACGTCGGCTTTGACAGC	*Bam*HI, F
WdCDC10. 2p	GGATCCGCTAAAGCCGACGTGGCTCCG	*Bam*HI, R
WdCDC10. 3p	GAATTCCAACGC**ATG**ACCATGAATGGATAT	*Eco*RI, F
WdCDC10. 4p	GAATTCATATCCATTCATGG**TCA**TGCGTTG	*Eco*RI, R
WdCDC10. 5p	GGATCCCTAGCATGGCAATGCCTCAGG	*Bam*HI, F
WdCDC10. 6p	TCTAGA***TCC***$^{++}$ATATCCATTCATGGTCAT	*Xba*I , (TAA→GGA), R
WdCDC10. 7p	GAATTCCATTGACTTGAAGCCGTCTG	*Eco*RI, F
WdCDC11. 1p	CGGGATCC**ATG**TTCTCTACTAATAAC	*Bam*HI, F
WdCDC11. 2p	GGGGATCC**TTA**TGCCTGTTGGTGGAC	*Bam*HI, R
WdCDC11. 3p	CGGGATCCATGCGATTGTCCGATGTGCAG	*Bam*HI, F
WdCDC11. 4p	GGATCCCACATTCTTCTTTCTCCGGAT	*Bam*HI, R
WdCDC11. 5p	GAATTCGATAAGCGACAAGAACTGCTG	*Eco*RI, F
WdCDC11. 6p	GAATTCCAGCAGTTCTTGTCGCTTATC	*Eco*RI, R
WdCDC11. 7p	GAACTAGTTTATGCCTGTTGGTGGAC	*Spe*I, F
WdCDC12. 3p	GCGGTACC**ATG**GCGCCTGCTCCCGTG	*Kpn*I, F
WdCDC12. 4p	CCGGTACC**TCA**GCTCCTGCGCACTCCT	*Kpn*I, R
WdCDC12. 5p	GGGGATCCATGGCGCCTGCTCCCGTG	*Bam*HI, F
WdCDC12. 6p	GGAAGCTTATAACGA CGACCGTGGCT	*Hind*III, R
WdCDC12. 7p	CCGCGGCGAAGCCACGGTCGTCGT***TAT***	*Sac*II (TAA→TAT), R

Table 2, cont.

WdCDC12. I1	GGAAGCTTACGACAATTAGAGGGTATG	*Hind*III, F
WdCDC12. I2	GGCTCGAGGCGATGGATGGTTCTGTCA	*Xho*I, R
WdCDC12. I3	AAGCGGCCGCGACTCTTGCATAGATGTC	*Not*I, F
WdCDC12. I4	AAGCGGCCGCTGAATTGGGACCTCGATC	*Not*I, R
Primers for WdChs5pMyo12 antibody production		
WdMyo12.1p	CGGAATTCGAGGAGAGGTACAATGGAAGA AGCGAAGGTCAGGCGG	*Eco*RI, F
WdMyo12.2p	CCGGATCCCTATCA**ATGGTGATGGTGATG** **GTG**ACGATCGCTCCCGACGACACCGCCGTC	*Bam*HI, R, 6X HIS-tag

[+] Degenerate nucleotide designations: D, A+T+G; H, A+C+T; R, A+G; Y, C+T; and N, any nucleotide.

* Sequences for restriction endonuclease recognition sites are underlined. ** Bold letters indicate start or stop codon. [++] Bold italic letters indicate stop codon point mutation. F: forward direction, R: reverse direction.

Table 3. Plasmids used for this study.

Plasmid	Description	Purpose
pCB1004	Chloramphenicol-resistant (Chl^r), f1 origin of replication (Ori), Hygromycin B-resistant (hph^r), a pBC-derived vector	*Wangiella*-specific disruption and transformation
pUC19	Ampicillin-resistant (Amp^r), Stratagene, USA	Cloning and sequencing
pBluescript II SK (+)	Amp^r, Stratagene, USA	As above
pGEM-T	Amp^r, Promega,	As above
pGEM-T easy	Amp^r, Promega, USA	As above
pCW2	Amp^r, f1 Ori, containing 508 bp *WdCDC10* PCR fragment in pGEM-T easy vector	Cloning
pCW3	Similar to pCW2, but containing 206 bp of *WdCDC12*	As above
pCW5	Similar with pCW2, but containing 766 bp of *WdCDC3*	As above
pCW6	Chloroamphenicol resistance (Chl^r), hph^r, *Eco*RI digestion fragment (~800 bp) of pCW5 was cloned into the *Eco*RI site of pCB1004.	Insertion disruption
pCW8	Similar to pCW6, but containing *Apa*I/*Spe*I digestion fragment (~500 bp) of pCW2; was cloned into the *ApaI*/*Spe*I sites of pCB1004.	As above
pCW9	Similar to pCE6, but containing *Eco*RI-digestion fragment (~250 bp) of pCW3; was cloned into the *Eco*RI site of pCB1004.	As above
pCW11	Similar to pCW2, but containing 610 bp of *WdCDC11*	Cloning

Table 3,cont.

pCW12	Similar to pCW6, but containing *Eco*RI digestion fragment (~610 bp) of pCW11; was cloned into the *Eco*RI site of pCB1004.	Insertion disruption
pCW18	*Chl*^{r}, ~3 Kb *Hind*III-digested genomic DNA containing full length of *WdCDC3* was cloned into the *Hind*III site of pCB1004.	Cloning
pCW19	*Amp*^{r}, ~3.2 Kb *Bam*HI-digested genomic DNA containing full length of *WdCDC10* was cloned into the *BamH*I site of pUC19.	As above
pCW20	*Chl*^{r}, ~5 Kb *Eco*RI-digested genomic DNA containing full length of *WdCDC11* was cloned into the *EcoR*I site of pCB1004.	As above
pCW21	*Amp*^{r}, ~7 Kb *Hind*III-digested genomic DNA containing full length of *WdCDC12* was cloned into the HindIII site of pBluescriptII SK (+).	As above
pCB1551	*Chl*^{r}, *sur*^{r}, containing ~2.8 Kb *sur* gene in *Sal*I site	Deletion mutagenesis
pCW29	*Amp*^{r}, *WdCDC3* cDNA amplified by RT-PCR using WdCDC3. 1p and WdCDC3. 2p as primers was cloned into pGEM-T easy.	Septin expression
pCW30	*Amp*^{r}, *WdCDC10* cDNA amplified by RT-PCR using WdCDC10. 3p and WdCDC10. 4p as primers was cloned into pGEM-T.	As above
pCW31	*Amp*^{r}, *WdCDC11* cDNA amplified by RT-PCR using WdCDC11. 1p and WdCDC11. 7p as primers was cloned into pGEM-T.	As above
pCW32	*Amp*^{r}, *WdCDC12* cDNA amplified by RT-PCR using WdCDC12. 3p and WdCDC12. 4p as primers was cloned into pGEM-T easy.	As above

Table 3, cont.

pRS415-*ADH1*	*Amp*^{r}, *LEU2*, CEN, containing *ADHI* promoter and *CYC1* terminator	*W. dermatitidis* septin cDNA expression
p3UD	*Chl*^{r}, *hph*^{r}, *Bam*HI/*Cla*I upstream fragment (~250 bp) of *WdCDC3* and *Hind*III/*Xho*I fragment of pCW18 were cloned into the *SpeI*/*Sac*II and *Hind*III/*Xho*I sites of pCB1004, respectively.	*WdCDC3* knock-out
pCW34	*Amp*^{r}, *sur*^{r}, 1.4 Kb *Eco*RI/*Sal*I fragments of up- and downstream *WdCDC10* were ligated with the *Xho*I/*Eco*RI fragment of pCB1551 (*sur*, ~2.8 Kb) and then were cloned into the *Bam*HI site of pUC19.	*WdCDC10* knock-out
pCW37	*Chl*^{r}, *hph*^{r}, the *Eco*RI/*Spe*I fragment (~600 bp, upstream) and the *Bam*HI/*Eco*RI fragment (~1 Kb, downstream) homology regions of *WdCDC11* were ligated and then were cloned into the *Bam*HI/*Spe*I sites of pCB1004.	*WdCDC11* knock-out
p12UD	*Chl*^{r}, *hph*^{r}, upstream (~450 bp, WdCDC12. I1 and WdCDC12. I2) and downstream (~200bp, WdCDC12. 7p and WdCDC12. I4) homology regions achieved by PCR of *WdCDC12* were cloned into the *Hind*III/*Xho*I and *Not*I/*Sac*II sites of pCB1004, respectively.	*WdCDC12* knock-out
pCW62	*Amp*^{r}, *sur*^{r}, the *Eco*RV/*Xho*I fragment of pCB1551 (*sur*, ~2.8 Kb) was ligated into the *Msc*I/*Sal*I sites of pCW29, which will derive a deletion of *WdCDC3* from +450 to +1,150.	*WdCDC3* partial deletion

Table 3, cont.

pCW63	*Amp*^{r}, *sur*^{r}, the *Pst*I/*Sac*II fragment of pCW31 was cloned into the corresponding sites of pCB1004 and then digested with *Pst*I/*Sac*I. This fragment was ligated into the corresponding sites of pUC19 and again digested with *Xho*I/*Sal*I, which finally was ligated with the *Sal*I fragment of pCB1551 (*sur*, ~2.8 Kb). This construct will derive a deletion of *WdCDC11* from +340 to +725.	*WdCDC11* partial deletion
pCW64	*Amp*^{r}, *sur*^{r}, similar to pCW62, but *Spe*I/*Xho*I fragment of pCB1551 (*sur*) was ligated into the *Avr*II/*Sal*I sites of pCW32. This will derive a deletion of *WdCDC12* from +390 to +750.	*WdCDC12* partial deletion

J. Sequencing and computer analysis

The nucleotide sequences of the *W. dermatitidis* septin genes were assigned the GenBank accession number DQ399319 for *WdCDC3*, DQ399320 for *WdCDC10*, DQ399321 for *WdCDC11*, and DQ399322 for *WdCDC12*. The *W. dermatitidis* septin genes were sequenced by the Institute of Cellular and Molecular Biology of the University of Texas at Austin. The deduced amino acid sequences of the *W. dermatitidis* septin genes were determined using the ExPASy translation tool (http://ca.expasy.org/tools/dna.html) and the NCBI Conserved Domain Search program (http://www.ncbi.nlm.nih.gov/Structure/cdd). The sequence comparisons among the fungal septin homologues were performed using the BLAST software system from NCBI and the Clustal W multiple-alignment tool available from the European Bioinformatics Institute (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

K. Transformations of *W. dermatitidis*, *S. cerevisiae*, and ***E. coli***

Early log-phase yeast cells grown at 26°C were used for the transformation of *W. dermatitidis*. The yeast cells were made by washing with ice–cold, 20 mM HEPES buffer (pH 7.0) containing 0.25% (V/V) 1 M DTT and 0.6 M sorbitol, followed by washing three additional times with ice–cold 1 M sorbitol. The final concentration of the competent cells was approximately 10^8 cells/ml. Before transformation, plasmids were linearized by the appropriate restriction enzymes and were repurified from gels using the QIAquick gel extraction kit (QIAGEN). About 1 μg of DNA was used with 10^8 competent cells (200 ml). Electroporation of the cell

suspensions was performed with a Gene Pulser® electroporation system with 0.2 mm cuvettes (BioRad, Richmond, CA) and using a condition of 1.45 KV field strength, a 25 μF capacitance, and a 200 Ω resistance. After 2–4 h incubation in 0.8-ml YPD at room temperature, the cell suspensions were spread on appropriate selection plates at 26°C for 3–7 days.

For *S. cerevisiae* transformations, cells were cultured to a density of 5 x 10^7 cells/ml at 26°C in 100 ml of YPD inoculated with 5 ml of an overnight culture of the strain to be transformed. After the cells were then harvested by centrifugation at room temperature at 4,000 rpm for 3 min, they were washed with 20 ml dH_2O, collected again and were resuspended in PLATE buffer (40% (v/v) PEG3350, 0.1 M lithium acetate, 1X TE) to give a cell density of 4 x 10^8 cells/ml. For the transformation itself, 0.5 ml of cells were suspended in a 1.5 ml Effendorf tube containing 100 μg of sonicated and boiled salmon sperm DNA (Sigma) as a carrier DNA, after which the plasmid DNA to be used for transformation was added into the cell suspension and mixed by short vortexing at a setting of 7. The transformation mixture was placed in a 30°C incubator and shaken for 30 min, and then 50 μl of DMSO was added and mixed by brief vortexing. After incubating at 42°C for 15 min with shaking every 5 min, 0.15 ml aliquot of transformation mixture was finally spread on appropriate selection media to identify transformants.

For transformation of *E. coli*, competent cells were prepared as follows: first, 0.5 ml of an overnight seed culture was inoculated into 50 ml fresh LB broth and incubated to $O.D._{600}$ 0.3; second, cells were chilled on ice for 15 min and collected by centrifugation at 4°C; third, the harvested cells were washed with 25 ml of cold TFB

buffer 1 (30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, and 15% (v/v) glycerol, pH 5.8), and chilled again on ice for 15 min and harvested by centrifugation at 4°C; fourth, the cells were finally resuspended with 2 ml of cold TBF buffer 2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, and 15% (v/v) glycerol, pH 6.5). For a transformation, 100 μl of competent cells were mixed with plasmid DNA, incubated on ice for 20 min and then heat shocked at 42°C for 60 sec. This transformation mixture was then incubated with 0.75 ml of fresh LB for 40 min at 37°C with shaking, after which cells were spread on appropriate plate media containing antibiotics.

L. The production of the polyclonal anti-WdChs5p rabbit antisera

To investigate the localization of WdChs5p during yeast budding, a DNA fragment that encodes the unique 108 amino acids located near the end of the myosin-motor like domain (WdChs5pMyo12) of WdChs5p was amplified by PCR. Primers (Table 2), WdMyo12 and WdMyo12.2p, carrying *Eco*RI and *Bam*HI restriction enzyme sites, respectively were used to derive a 6x*HIS*-tagged PCR product. The resulting PCR product was then cloned into the *Eco*RI/*Bam*HI sites of the pLM1 vector carrying the T7 promoter (Abramczyk *et al*., 2004), and used to transform *E. coli* strain BL21. Once growth reached $O.D._{600}$ 1.0 at 37°C, the expression of the WdChs5pMyo12 domain was induced by adding IPTG (1mM) directly to the culture and incubating for an additional 4 h. For immunization of New Zealand rabbits (Pacific Immunology Co., Ramona, CA), inclusion body fractions were purified under denaturating condition using Ni-NTA chromatography (Qiagen Inc., USA, data not

shown). The specificity of anti-WdCh5pMyo12 serum was tested by Western blot analysis of membrane proteins isolated from *W. dermatitidis* cells (Liu *et al*., 2004). For immunoblotting, the serum was diluted (1:5,000).

M. Photomicroscopy

For photomicroscopy, *W. dermatitidis* cells were cultured at both 26°C and 37°C and were sampled every 3 h, periodically depending upon the experiment, followed by the formaldehyde fixation (final 5% conc.) for 2 h. The fixed cells were washed with 1X PBS. For nucleus and chitin staining, cells were stained with 1X PBS buffer containing 5 µg/ml DAPI (4,6-diamidino-2-phenylindole) (Sigma) and 20 µg/ml Calcoflour white, respectively, for 10 min and then washed three times with 1X PBS.

For WdChs5p immunocytology, spheroplasts were prepared by incubating cells with zymolyase (1 µg/ml, ZYMO Research, USA) in a spheroplast buffer [0.9 M sorbitol, 0.1 M EDTA, 1% (V/V) beta-mercaptoethanol] for 1 h at 30°C. The spheroplasted cells were then incubated with anti-WdChs5p rabbit antisera (1:100) in 1X PBS buffer containing 1 mg/ml BSA and 0.03% (V/V) NaN_3 for 2 h, then washed three times with 1X PBS buffer, and finally incubated with the anti-rabbit goat antibody (FITC, 1:250) in 1X PBS buffer containing 1 mg/ml BSA and 0.03% (V/V) NaN_3 for 1 h, prior to washing the cells three times with 1X PBS buffer. To investigate whether WdChs5p localization was associated with microtubules during yeast growth, WT cells were cultured in two ways in the presence of nocodazole (final conc. 20 µg/ml, Sigma, St Louis, MO), a known inhibitor if microtubule polymerization in *W.*

dermatitidis (Jacobs and Szaniszlo, 1982), overnight at 37°C. In the first, yeast were harvested and fixed for 2 h with formaldehyde (final 5% conc.), while in the second, cells were harvested and washed 3 times with 1X PBS to remove nocodazole from the medium, re-cultured at 37°C in pre-warmed, fresh YPD broth, and sampled every 30 min and fixed for 2 h with formaldehyde (final 5% conc.). To investigate whether actin is associated with WdChs5p localization during yeast growth, latrunculin A (LAT-A, final conc. 200 μM, Biomol Research Labs, Inc., Plymouth Meeting, PA), an actin polymerization inhibitor (Ayscough *et al*., 1997), was sequentially added at 10 min intervals to 12 cultures (37°C) containing ~1 X 10^7 stationary-phase WT cells, each of which consisted of about 90% of unbudded cells (Roberts and Szaniszlo, 1980) grown at 26°C, which were then incubated for additional 10 min prior to formaldehyde fixation (final 5% conc.) for 2 h. The fixed cells were then treated with WdChs5p antibody, stained with FITC-conjugated anti-rabbit goat antibody and DAPI as described above, and finally examined and photographed with a Zeiss Axio imager A1 microscope system (Zeiss, Germany).

N. Northern blot analysis

The upstream homology regions of *WdCDC11* used for the integration disruption (Figure 15A) and the *WdCDC12* deletion (Figure 15A) used as probes for the Northern blot analysis were labeled according to the manufacture's instruction (Ambion, Austin, TX). *W. dermatitidis* cells cultured in YPD broth for 3 days were harvested and then washed with dH_2O. Total RNA was isolated from spheroplasts as described in section E. RNA electrophoresis was performed under denaturing

conditions as described previously (Liu *et al*., 2004), using a MOPS buffer system containing 2.2 M formaldehyde. Shortly thereafter, 10 µg RNA (approximately 5 µl) was added into 15 µl RNA denaturating buffer, the mixture heated for 5 min at 68°C, then chilled on ice for 10 min, after which 4 µl RNA loading buffer and 0.5 µl ethidium bromide (Sigma, 0.5 mg/ml) were added. Following electrophoresis, the gel was rinsed with RNase-free water and transfer buffer (20X SSC), and the RNA transferred to the nitrocellulose membrane. Later steps were performed as described in section F above.

III. RESULTS

1. Isolation and characterization of *W. dermatitidis* septin genes.

Because all septins have a well conserved GTP binding domain, the amino acid sequences in that domain were identified and used to design degenerate primers, which were used to amplify fragments of four different *WdCDC* septin genes (Table 4). Each full-length gene was then isolated from sub-genomic libraries, after screening by Southern blotting using the PCR products as probes (Figure 11). Sequence analysis showed that all four septin genes had at least one intron: one in *WdCDC3,* three each in *WdCDC10* and *WdCDC11,* and two in *WdCDC12* (Table 5). The analysis also showed that each deduced septin protein (WdCdcp) had highest amino acid identity with its orthologs in *Aspergillus* species and least with its orthologs in *S. cerevisiae* (Table 5 and Figure 12). In addition, and consistent with results concerning other fungal septin proteins, those of *W. dermatitidis* showed the three expected major conserved domains; an N-terminus polybasic domain, a GTP-binding domain, and with the exception of WdCdc10p a predicted C-terminus coiled-coil region (Figure 13).

To determine if the cloned *WdCDC* septin genes were functional homologs of those in *S. cerevisiae*, the cDNA of each was expressed in a corresponding *S. cerevisiae* ts *CDC* mutant. Even with their relatively low homologies, the cDNA of *WdCDC3* (totally) and *WdCDC12* (partially) complemented the corresponding *S. cerevisiae* mutation (Table 6). Furthermore, the expression of *WdCDC3* in *S. cerevisiae cdc3-6* not only corrected its ts growth defect, but also its morphological defect at the restrictive temperature (Figure 14). However, neither the introduction of the cDNA of *WdCDC10*

Table 4. The primers used for obtaining the *W. dermatitidis* septin gene fragments.

Gene		Conserved amino acid sequence	Degenerative primers (5' → 3')
			Nucleotide sequences[+]
WdCDC3	F[*]	**DIEENGV**	GAYATHGARGARAAYGGNGTN
	R[*]	**VFQQKVA**	NGCNACYTTYTGYTGRAANAC
WdCDC10	F	**PIVKYIK**	CCNATHGTNAARTAYATHAAR
	R	**QDLIETT**	NGTNGTYTCDATNARRTCYTG
WdCDC11	F	**DTPGFG**	GAYACNCCNGGNTTYGGN
	R	**YENYRT**	NGTNCKRTARTTYTCRTA
WdCDC12	F	**DTPGFG**	GAYACNCCNGGNTTYGGN
	R	**IPVIAKA**	NGCYTTNGCDATNACNGGDAT

[*] F and R indicate forward and reverse direction of primers, respectively. [+] Degenerate nucleotide designations: [+] Degenerate nucleotide designations: D, A+T+G; H, A+C+T; R, A+G; Y, C+T; and N, any nucleotide.

Figure 11. Sub-genomic libraries of each septin gene. Constructed as previously described (Liu *et al*., 2004). Arrows identify gene signals for the A) ~3 Kb *Hind*III-fragment for *WdCDC3*, B) ~5.5 Kb *Eco*RI-fragment for *WdCDC10*, C) ~5 Kb *Eco*RI-fragment for *WdCDC11*, D) ~7 Kb *Hind*III-fragment for *WdCDC12*.

Figure 11

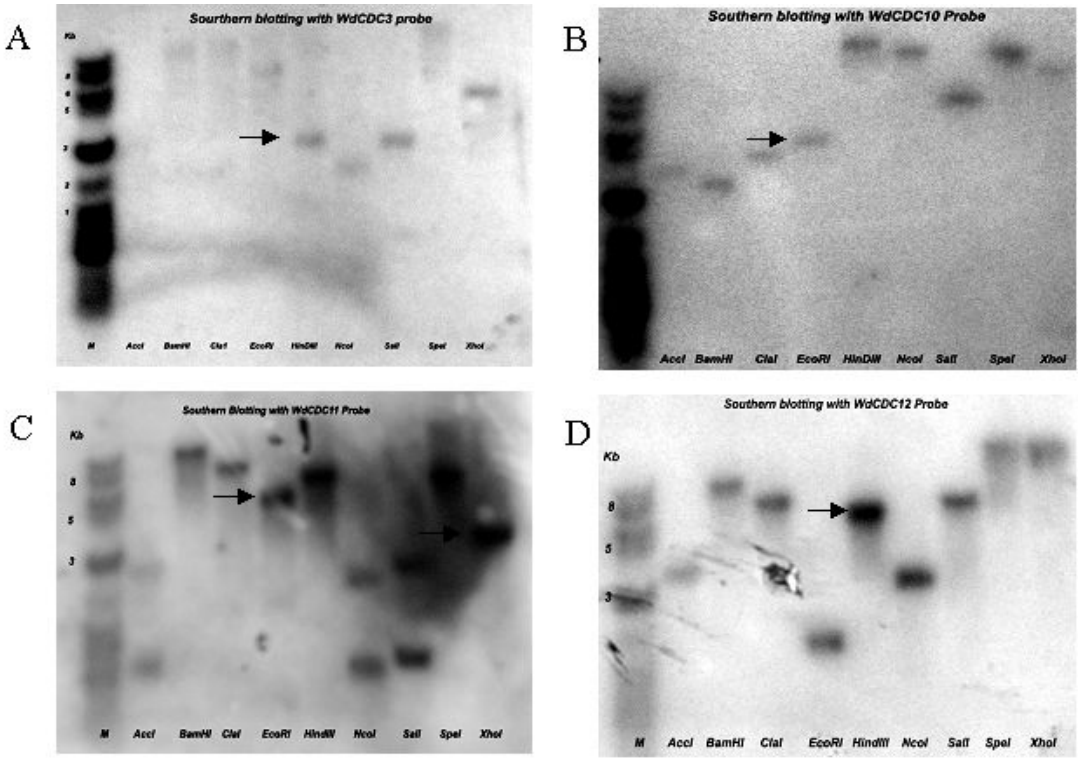

A
Sourthern blotting with WdCDC3 probe
Kb
M Acci BamHI ClaI EcoRI HinDIII NcoI SalI SpeI XhoI
B
Southern blotting with WdCDC10 Probe
AcciI BamHI ClaI EcoRI HinDIII NcoI SalI SpeI XhoI
C
Southern Blotting with WdCDC11 Probe
Kb
8
5
3
M Acci BamHI ClaI EcoRI HindIII NcoI SalI SpeI XhoI
D
Southern blotting with WdCDC12 Probe
Kb
8
5
3
M AcciI BamHI ClaI EcoRI HindIII NcoI SalI SpeI XhoI

Table 5. Homology of the four *W. dermatitidis* septin genes with other fungal septins.

Septin gene	Introns	Sizes (a.a.)**	Homology *	
			Highest	Lowest
WdCDC3	1	1279 bp (407)	*Aspergillus nidulans* AspB I#: 91% S+: 94%	*Saccharomyces cerevisiae* Cdc3p I: 52% S: 68%
WdCDC10	3	1161 bp (339)	*A. fumigatus* Septin I: 80% S: 83%	*S. cerevisiae* Cdc10p I: 52% S: 68%
WdCDC11	3	1337 bp (390)	*A. nidulans* AspA I: 95% S: 98%	*S. cerevisiae* Cdc11p I: 55% S: 74%
WdCDC12	2	1258 bp (385)	*A. nidulans* AspC I: 87% S: 92%	*S. cerevisiae* Cdc12p I: 56% S: 71%

* Only the GTP binding domains of the identified proteins were used for the homology comparisons. ** a.a: amino acid. # I: identity, +S: similarity of the amino acid level by NCBI BLAST

Figure 12. Phylogenetic tree of *W. dermatitidis* and other fungal septins. Sequences were generated and aligned using the CLUSTAL W program at http://www.ebi.ac.uk/clustalw. Ag: *A. gossypii*, An: *A. nidulans*, Ca: *C. albicans*, Sc: *S. cerevisiae*, Sp: *S. pombe*, Wd: *W. dermatitidis*.

Figure 12

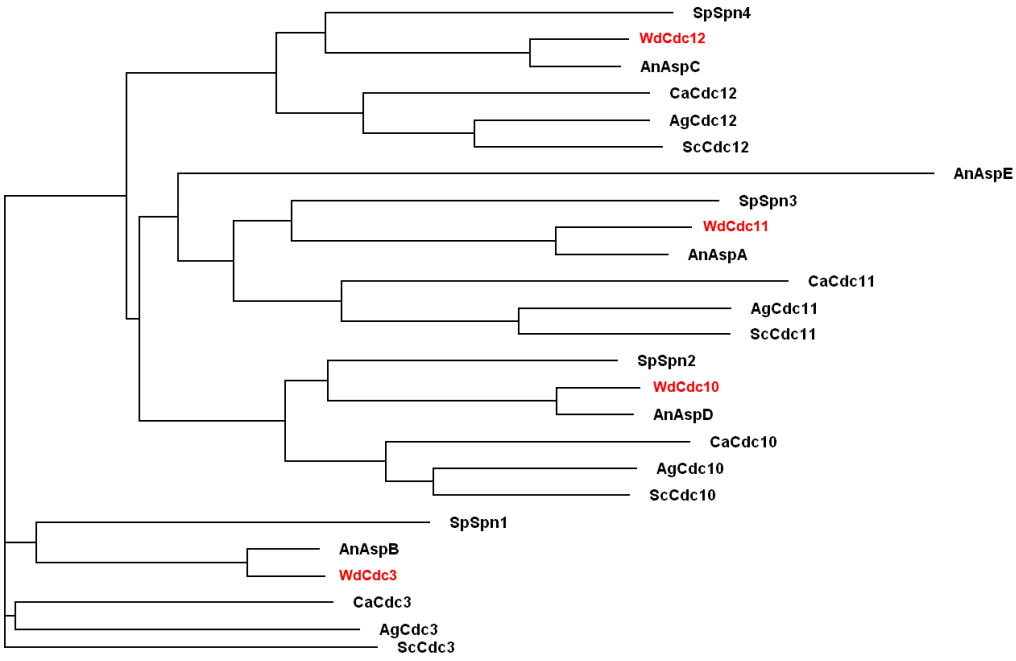

SpSpn4
WdCdc12
AnAspC
CaCdc12
AgCdc12
ScCdc12
AnAspE
SpSpn3
WdCdc11
AnAspA
CaCdc11
AgCdc11
ScCdc11
SpSpn2
WdCdc10
AnAspD
CaCdc10
AgCdc10
ScCdc10
SpSpn1
AnAspB
WdCdc3
CaCdc3
AgCdc3
ScCdc3

Figure 13. Alignment of the deduced amino acid sequences* of the *W. dermatitidis* septins. * The deduced amino acids sequences were obtained with the ExPASy translation tool (http://ca.expasy.org/tools/dna.html) and the deduced domains were identified with NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd). The letters in a box identify the polybasic amino acids domain (PB); the bold letters identify the conserved GTP binding domain; the letters in grey identify the C-terminus expected coiled-coil region which is absence in WdCdc10p.

Figure 13


WdCdc3p MRNVVRRKLTGYVGFANLPNQVHRKSVRKGFNFNVMVVGESGLGKSTLVNTLFNTSLYPP 60
WdCdc10p MPQGDAVFPRSHVGFDSITSQIFKKLLKRGFQFNIICVGQTGLGKSTLINTIFASHLIDS 60
WdCdc11p ------MFSTNNMRLSDVQLRRKKIVK-KGIQFCLMVCGASGTGRTTFVNTLCGKRVLQP 53
WdCdc12p -MAPAPVESASPIGIANLPNQRHKIVAKRGAAFTIMVAGESGLGKTTFINTLFSTTIKNY 59
WdCdc3p RERKGPSLDIIPKTVSIQSIS--ADIEENGVRLRLTVVDTPGFGDFVNNDDSWRPIVENI 118
WdCdc10p KGRLRPD-EPVRATTEIQTVSH-VIIEENGVRLRLNIVDTPGYGDLVNNDRCWDPIVKYI 118
WdCdc11p KDADDAANAHLEEGVRIKPVTVGLELDEEGTRVSLTIVDTPGFGDQIDNEASFGEIVGYL 113
WdCdc12p QDHKRRHAKQVDKTVEIEITKA--ELEEKFFKVRLTVIDTPGFGDYVNNRDSWMPIIEFL 117
WdCdc3p EQRYDAYLEAENKVNR-MNIVDNRIHACVYFIQPTGHSLKPLDIRVMRRLHTKVNPIPVI 177
WdCdc10p KDQHSAYLRKELTAQRERYIQDTRIHCCLFFIQPSGHALKPLEIVVLKKLSDVVNVVPVI 178
WdCdc11p ERQYDDILAEESRIKRNPRFRDNRVHALLYFITPTGHGLRELDIELMKRLSPRVNVIPVI 173
WdCdc12p DDQHESYMLQEQQPRRQDKI-DLRVHACLYFIRPTGHTLKPLDIEVMKRLCTRVNLIPVV 176
WdCdc3p AKADTLTDEEVAAFKARARILADIQHHSIQIFEGP-HYELDDEETIAENQEIMSKVPFAV 236
WdCdc10p AKADSLTLEERRAFKER--IKEEFAFHNLKMYPYD-NDELDDEE-RATNATIKDIIPFAV 234
WdCdc11p GKADSLTPAELAESKKL--VMEDIEHYRIPVYNFPYDIEEDDEDTVEENAELRGLMPFAI 231
WdCdc12p AKADTLTPADLARFKHR--IRNVIEAQGIKIYQPP--IEDDDEAAVQHARALIAAMPFSV 232
WdCdc3p VGANYEVTNAEGRKVRGRRYPWGIIEVDNEDHCDFVKLRQMLIRTHMEELKEHTNNTLYE 296
WdCdc10p VGSEKSIIVN-GKQVRGRQTKWGVINVEDENHCEFVYLRNFLTRTHLQDLIETTSQIHYE 293
WdCdc11p VGSE-DVLEIGGRRVRARQYPWGVVEVDNPRHSDFLAIRSALLHSHLADLKEITHDFLYE 290
WdCdc12p IGSEKDVKTPDGRVAKGRQYIWGVAEVENEEHCDFKKLRSILIRTHMLDLIHTTEENHYE 292
WdCdc3p NYRSDKLIQMGVSQDPSV--FKEVNPAVKQEEERTLHEQKLAKMEAEMKMVFQQKVQEKE 354
WdCdc10p TFRAKQLLALKES SAASGMGGSRPISPAADR---ELSRGSQRMTMN--------GY--- 338
WdCdc11p NYRTEKLSKSVDGASRGQ--DSSINPEDLASQSVRLKEEQLRREEEKLREIEVKVQREIN 348
WdCdc12p AYRAQQMETRKFGEARPRKLDNPKFKEEEEALRKRFT-EQVKVEEQRFRAWEQKLIAERD 351
WdCdc3p SKLQQSEEELFARHREMKEQLERQRHELEEKKARIEQGRPIEKEGKRKGFSLR 407
WdCdc10p ---------- 338
WdCdc11p DKRQELLGCGRANSGEIQAWMPPRTKPVSGRTSTATVHQQA---------- 389
WdCdc12p RLNKDLEATHAAIK-----QLEQELEQMQG--SAVRSHGRR---------- 385

Table 6. Heterologous complementation of *S. cerevisiae* septin ts mutant strains with corresponding *W. dermatitidis* septin cDNAs.

Strain	Temperature sensitivity (ts)				Morphology *
	26 °C	30 °C	35 °C	37 °C	
Wild type (WT)	+++#	+++	+++	+++	Yeast
cdc3-6	+++	+	-†	-	Elongated buds**
cdc3-6WdCDC3	+++	+++	+++	+++	Yeast
cdc10-1	+++	-	-	-	N.D
cdc10-1WdCDC10	+++	-	-	-	N.D
cdc11-6	+++	-	-	-	N.D
cdc11-6WdCDC11	++	-	-	-	N.D
cdc12-6	+++	+	-	-	Elongated buds
cdc12-6 WdCDC12	+++	+++	-	-	Elongated buds

Each *W. dermatitidis* septin gene was cloned into pRS415 containing the *ADH1* promoter and then transformed into the corresponding *S. cerevisiae* ts mutant. The selected transformants were spotted on YPD and SD agar medium lacking Leu. *, morphology at restrictive temperature (37°C); #, the + number indicates the amount of growth compared to that of the wild type strain; †, no growth; **, elongated buds with cytokinesis defects (Lee *et al*., 2002). N.D, not determined.

nor *WdCDC11* rescued the ts phenotypes of the corresponding *S. cerevisiae cdc10* and *cdc11* septin mutants (Table 6).

2. Characterization of *W. dermatitidis* septin gene function by mutagenesis.

To determine whether septins are important for viability, normal growth rates, and cellular development in *W. dermatitidis*, each *WdCDC* septin gene was disrupted by either replacement or by insertional mutagenesis with the antifungal markers, *sur* (for sulfonylurea resistance) or *hph* (for hygromycin resistance) (Figure 15 and 16). Because only an insertional disruption mutant (*wdcdc11*Δ*-1*) and a GTPase domain deletion mutant (*wdcdc12*Δ*-1*) were successfully derived in the haploid strain of *W. dermatitidis*, *WdCDC11* and *WdCDC12* were judged to be essential. Support for this conclusion was provided by finding that no haploid *wdcdc11*Δ and *wdcdc12*Δ mutant strains could be recovered after the deletion of one *WdCDC11* or *WdCDC12* gene in a parasexually-derived diploid strain (3u2m-428, Figure 17) following treatment with methyl benzimidazole-2-yl-carbamate to induce haploidization as described previously (Cooper and Szaniszlo, 1993; Ye and Szaniszlo, 2000). In contrast, *WdCDC3* and *WdCDC10* were judged to be nonessential because haploid strains with each gene deleted were successfully derived (Figure 15B). These results contrast with those of previous reports that showed *CDC3* (Longtine *et al*., 2000), *CaCDC3* (Warenda and Konopka, 2002), *SPN2* (An *et al*., 2004), and *aspB* (Momany *et al*., 2001) are essential genes, whereas *CDC11* (Longtine *et al*., 2000), *aspA* (Momany *et al*., 2001), and *SPN3* (An *et al*., 2004) are non-essential genes.

Figure 14. Phenotypic characteristics of the *S. cerevisiae cdc3-6* ts mutant heterologously expressing the *W. dermatitidis WdCDC3* cDNA. (A) Four *S. cerevisiae* transformants spotted on the agar media, YPD and SD supplemented with His, Lys, Trp, and Ura and cultured at 26, 30, and 37°C for 2 days. (B) Growth of strains incubated in YPD broth and studied in a time course manner. After overnight culture, the strains were harvested at 1,050 x *g* for 10 min, washed with 1X PBS, and ~$5x10^5$ cells used to inoculate pre-warmed 50-ml YPD media subcultures, which were incubated at 26 and 37 ºC. Samples (1 ml) were taken every 2 h for 12 h and O.D. at 595 nm determined. ■, 26°C WT; ▲, 26°C *cdc3-6*; ●, 26°C *cdc3-6* w/ *WdCDC3*; □, 37°C WT; Δ, 37°C *cdc3-6*; ○, 37°C *cdc3-6* w/ *WdCDC3*. C) Morphology of cells from B cultured for 8 h, then fixed with 5% formaldehyde (final concentration) and washed with 1X PBS prior to light microscopy. Bars equal 10 µm.

Figure 14

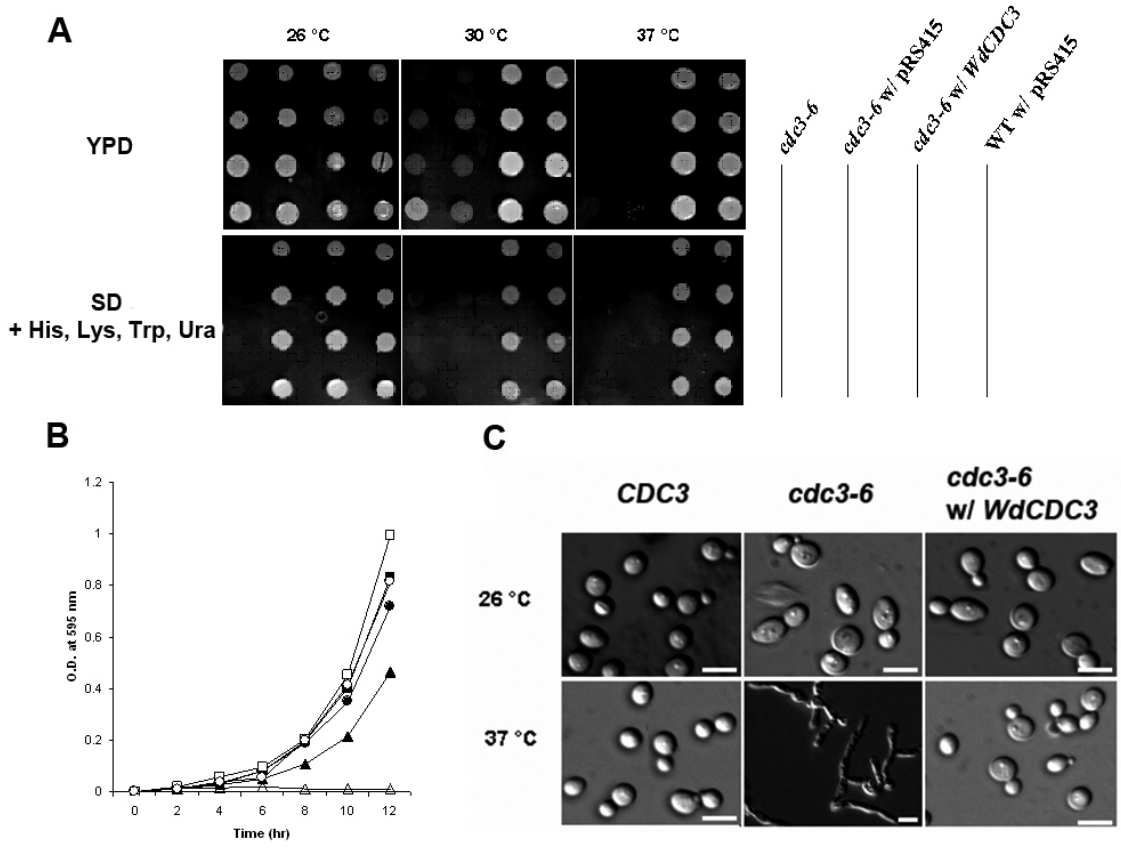
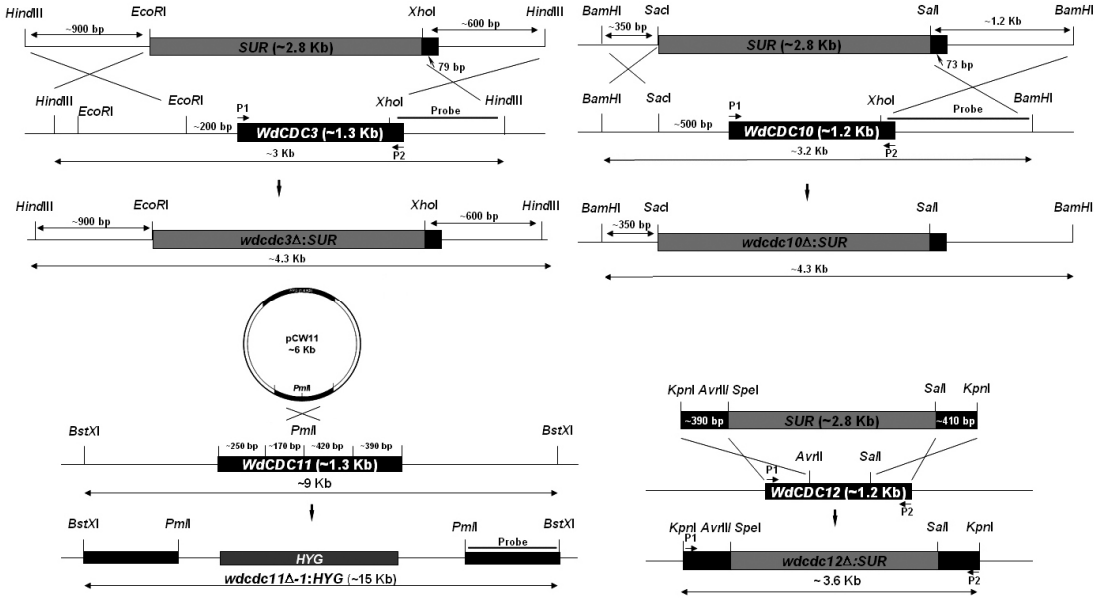

A
26 °C
30 °C
37 °C
YPD
SD
+ His, Lys, Trp, Ura
cdc3-6
cdc3-6 w/ pRS415
cdc3-6 w/ WdCDC3
WT w/ pRS415
B
O.D. at 595 nm
Time (hr)
C
CDC3
cdc3-6
cdc3-6
w/ WdCDC3
26 °C
37 °C

Figure 15. Mutagenesis of the *WdCDC* septin genes and Southern blot confirmation of the deletion or integrative disruption of each *WdCDC* gene. (A) Mutagenesis strategy of each *WdCDC* septin gene. (B) Southern blots: Lane 1, WT; lane 2, heterologous diploid *wdcdc3*Δ-*2*; lane 3, *wdcdc3*Δ-*2*; lane 4, WT; lane 5, heterologous diploid *wdcdc10*Δ -*1*; lane 6, *wdcdc10*Δ -*1*; lane 7, WT; lane 8, heterologous diploid *wdcdc11*Δ-*1*; lane 9, *wdcdc11*Δ-*1*; lane 10, WT; lane 11, *wdcdc12*Δ -*1*; lane 12, heterologous *wdcdc12*Δ -*1*. DNA concentration used for Southern blotting was 4 μg/μl.

Figure 15

A.


HindIII
EcoRI
XhoI
HindIII
~900 bp
~600 bp
SUR (~2.8 Kb)
79 bp
HindIII
EcoRI
EcoRI
P1
XhoI
Probe
HindIII
~200 bp
WdCDC3 (~1.3 Kb)
~3 Kb
P2
BamHI
SacI
SalI
BamHI
~350 bp
~1.2 Kb
SUR (~2.8 Kb)
73 bp
BamHI
SacI
P1
XhoI
Probe
BamHI
~500 bp
WdCDC10 (~1.2 Kb)
~3.2 Kb
P2
HindIII
EcoRI
XhoI
HindIII
~900 bp
~600 bp
wdcdc3Δ::SUR
~4.3 Kb
BamHI
SacI
SalI
BamHI
~350 bp
wdcdc10Δ::SUR
~4.3 Kb
pCW11
~6 Kb
PmlI
BstXI
PmlI
BstXI
~250 bp
~170 bp
~420 bp
~390 bp
WdCDC11 (~1.3 Kb)
~9 Kb
BstXI
PmlI
PmlI
BstXI
Probe
HYG
wdcdc11Δ-1::HYG (~15 Kb)
KpnI AvrII/ SpeI
SalI
KpnI
~390 bp
SUR (~2.8 Kb)
~410 bp
AvrII
SalI
P1
WdCDC12 (~1.2 Kb)
P2
KpnI AvrII/ SpeI
SalI
KpnI
P1
wdcdc12Δ::SUR
~ 3.6 Kb
P2

B.

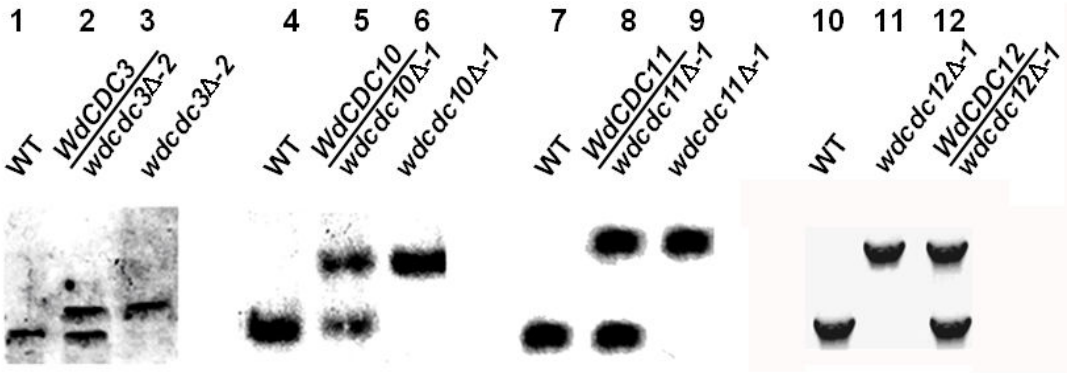

1
2
3
4
5
6
7
8
9
10
11
12
WT
WdCDC3
wdcdc3Δ-2
wdcdc3Δ-2
WT
WdCDC10
wdcdc10Δ-1
wdcdc10Δ-1
WT
WdCDC11
wdcdc11Δ-1
wdcdc11Δ-1
WT
wdcdc12Δ-1
WdCDC12
wdcdc12Δ-1

Figure 16. Disruption of the *WdCDC* septin genes. (A) RT-PCR confirmation of the deletion or integrative disruption of each *WdCDC* gene. M: 1 kb-ladder (NEB), Lane 1: WT total RNA with WdCDC3.1p/WdCDC3.2p, Lane 2: *wdcdc3*Δ*-1* total RNA with WdCDC3. 1p/WdCDC3.2p, Lane 3: *wdcdc3*Δ*-1* total RNA with WdCDC10.1p/WdCDC10.2p, Lane 4: WT total RNA with WdCDC10.1p/WdCDC10.2p, Lane 5: *wdcdc10*Δ*-1* total RNA with WdCDC10.1p/WdCDC10.2p, Lane 6: *wdcdc3*Δ*-1* total RNA with WdCDC3.1p/WdCDC3.2p, Lane 7: WT total RNA with WdCDC11.1p/WdCDC11.2p, Lane 8: *wdcdc11*Δ*-1* total RNA with WdCDC11.1p/WdCDC11.2p, Lane 9: *wdcdc3*Δ*-1* total RNA with WdCDC12.3p/WdCDC12.4p, Lane 10: WT total RNA with WdCDC12.3p/WdCDC12.4p, Lane 11: *wdcdc12*Δ*-1* total RNA with WdCDC12.3p/WdCDC12.4p, Lane 12: *wdcdc12*Δ*-1* total RNA with WdCDC11.1p/WdCDC11.2p. (B-D) Northern blot confirmation of the integrative disruption or deletion of the *wdcdc11*Δ*-1* and *wdcdc12*Δ*-1* strains. 1: WT, 2: *wdcdc11*Δ*-1*, 3: *wdcdc12*Δ*-1*. (B) Total RNAs before membrane transfer. (C) Total RNAs on the membrane. (D) Northern blot results: note no signals from both septin mutant strains.

Figure 16

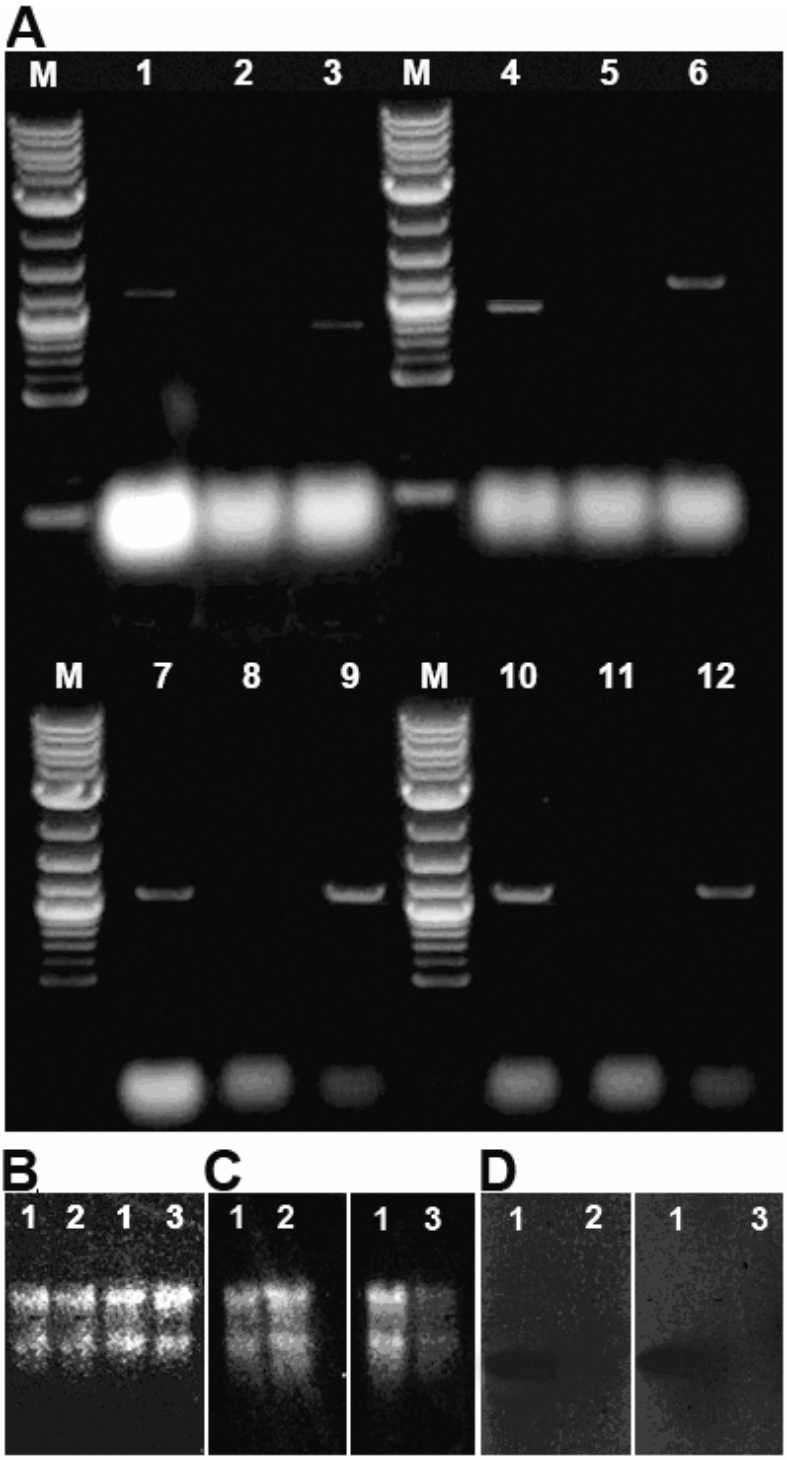
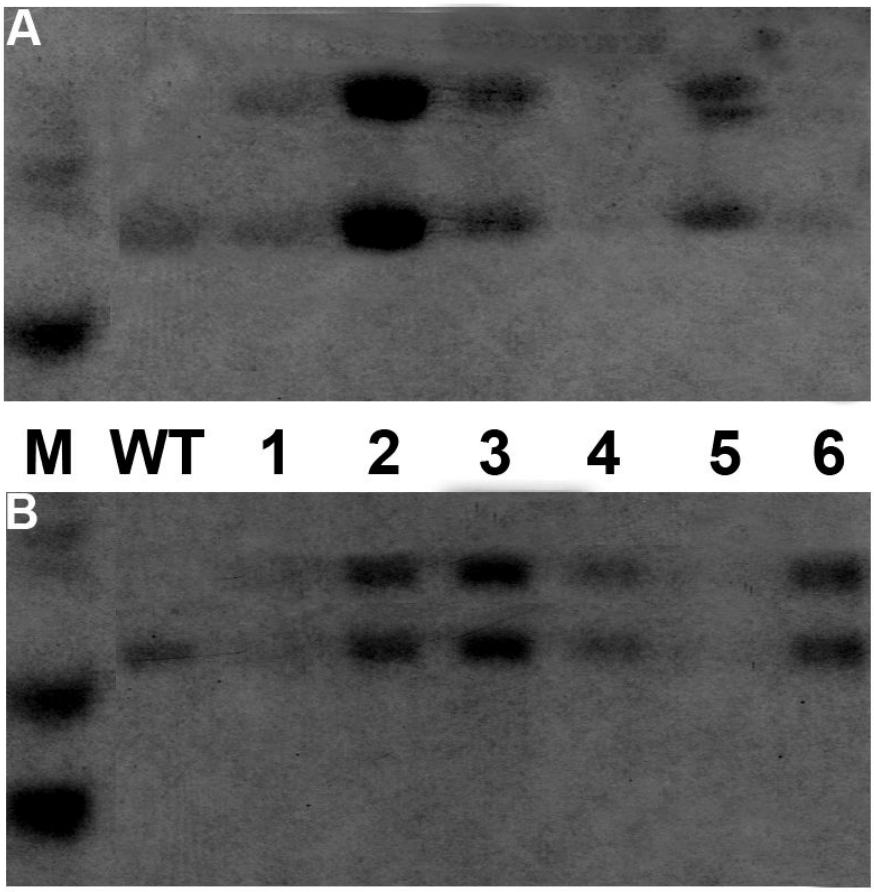


Figure 17. Southern blotting confirmation of the deletion of one *WdCDC11* or *WdCDC12* gene in a parasexually-derived diploid strain. (A) Genomic DNAs of the wild type (WT) and heterologous diploid strains (*wdcdc11*Δ/*WdCDC11*) were digested with *Eco*RI. (B) Genomic DNAs of the WT and heterologous diploid strains (*wdcdc12*Δ/*WdCDC12*) were digested with *Nco*I. Lane M: NEB 1 Kb-ladder, lane WT: WT (diploid, 3u2m-428), lane 1-6: the heterologous diploid strains.

Figure 17



Compared to WT, the knockout mutant *wdcdc3*Δ-*2* cultured on agar medium or in broth medium grew more poorly at both 26°C and 33°C and almost not at all at 37°C (Figure 18A). Also, *wdcdc3*Δ-*2* showed aberrant bud profiles compared with those of WT, with mother cells having two to four bud cells (Figure 18B) at both temperatures, whereas WT consisted mainly of yeast without buds or with only one bud. After a temperature shift to 37°C, by 12 h about 71% of the cells were dead (Table 7) as judged by tryphan blue staining. Furthermore, a "bursting balloon-like" phenotype often appeared (Figure 18B g and h), which was not corrected with 1M sorbitol (data not shown). Moreover, while this mutant frequently also exhibited aberrant septum positioning when cultured on agar media at 26°C (Figure 18C), which led to the production of the sclerotic morphotypes called planate cells, its nuclear distribution was normal (Figure 19). Interestingly, even though most cells of a mutant with an insertion in *WdCDC3* (*wdcdc3*Δ-*1*) also lost their normal yeast form and tended to form yeast cell chains, they showed no apparent growth defect when grown at 37°C (Figure 20). Collectively, these data indicated that *WdCDC3* is critical for sustaining viability, normal cell cycle progression, cell wall integrity, proper morphology, and normal septum formation in *W. dermatitidis*.

In contrast to the cells of the *wdcdc3*Δ-*2* knock-out strain, those with the *wdcdc10*Δ-*1* knock-out displayed no overall growth defect (Figure 20), even though they frequently had wider than normal bud necks than the WT (Figure 21C) and had multiple buds, which were not easily separated either by zymolyase treatment (1 mg/ml) or harsh

Figure 18. Growth and cellular morphology of the wild-type (WT) and the *wdcdc3*Δ-*2* strains. (A) Growth (O.D.) in YPD broth of the WT and *wdcdc3*Δ-*2* incubated at 26 and 37°C in a time course manner. ▲: WT, ■: *wdcdc3*Δ-*2*. (B) *wdcdc3*Δ-*2* yeast cells grown at 26°C and sampled and photographed in a time course manner during a YPD broth culture. a: 0 h, b-d: 3 h, e and f: 6 h, g and h: 9 h: arrows indicate bursting cells. (C) Phenotypes of WT, a heterozygous diploid, and the *wdcdc3*Δ-*2* strain grown on YPD agar medium and cultured at 26°C. Bars equal 10 μm.

Figure 18

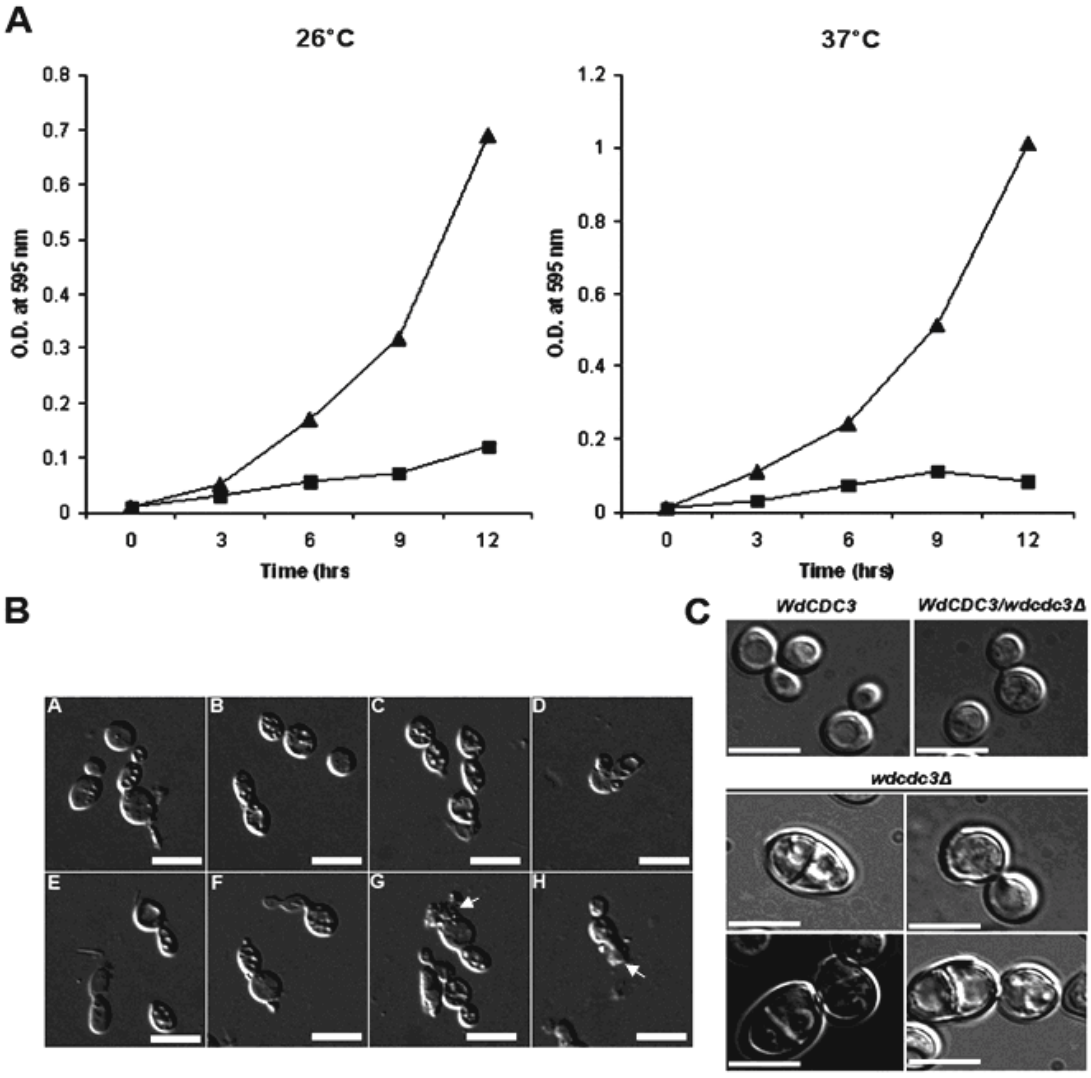

A
26°C
37°C
O.D. at 595 nm
Time (hrs
B
C
WdCDC3
WdCDC3/wdcdc3Δ
wdcdc3Δ

Table 7. Morphological differences between the wild type (WT) and each septin mutant cultured at 37 °C

Time	Strain	Yeast Morphology (%)									
		No bud		Small bud	Large bud						
		N*	abN+		Total	N	w/ wide bud neck	Multi-nuclei	w/ three buds	four or more buds	Dead
0 h	WT	37.7	0	32.5	29.8	29.8	0	0	0	0	0
	wdcdc3Δ-2	20.8	0	26.7	52.5	52.5	0	0	0	0	0
	wdcdc10Δ-1	37.3	0	25.4	37.3	29.7	**7.6**	0	0	0	0
	wdcdc11Δ-1	20.1	0	15.9	64	48.3	0	0	14.2	1.5	0
	wdcdc12Δ-1	17.9	7.1	3.6	54.3	16.4	0	**12.1**	12.9	19.3	**10.0**
12 h	WT	27.2	0	25.2	47.6	47.6	0	0	0	0	0
	wdcdc3Δ-2	5.2	0	8.3	**86.5**	15.3	0	0	0	0	**71.2**
	wdcdc10Δ-1	26.1	0	16.0	57.9	17.6	**15.1**	0	25.2	0	0
	wdcdc11Δ-1	11.8	0	3.1	**85.1**	4.7	0	0	24.0	43.1	**13.3**
	wdcdc12Δ-1	10.2	4.5	4.2	81.1	7.3	**Big$** 6.1	**11.6**	18.1	24.2	**13.8**
24 h	WT	73.6	0	10.8	**15.6**	13.0	0	0	2.6	0	0
	wdcdc3Δ-2	NA**	NA	NA	NA	NA	NA	NA	NA	NA	NA
	wdcdc10Δ-1	16.3	0	7.3	**76.4**	8.6	**16.4**	0	40.2	11.4	0
	wdcdc11Δ-1	6.2	0	1.2	**92.6**	6.3	0	0	23.3	31.8	**31.7**
	wdcdc12Δ-1	6.5	6.5	4.3	**82.7**	2.2	**Big 8.7**	**12.5**	17.4	26.1	**15.8**

Values are the averages of the three independent counts of the 100 cellular units.

* N: normal phenotype. + abN: major abnormal phenotypes including dead and large cells. $ Big: at least two times bigger than the WT cells.

Figure 19. Nuclei and chitin distribution in the *wdcdc3*Δ-*2* knock-out strain. A-E: DIC, F-J: DAPI and calcoflour co-staining of A-E, respectively. The *wdcdc3*Δ-*2* strain was grown on the YPD agar medium at 26°C. Bars equal 10 μm.

Figure 19

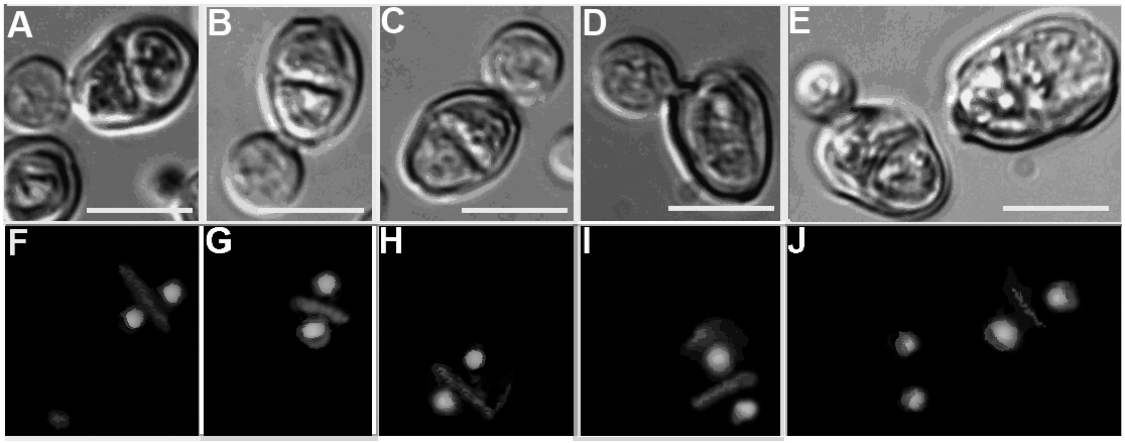


Figure 20. Growth at three temperatures of the wild-type (WT) and each septin mutant. Ten-fold serial dilutions of each strain (starting inoculum 1 x 10^7 cells) were spotted on YPD plates and incubated at 26, 33, and 37°C for 3 days.

Figure 20

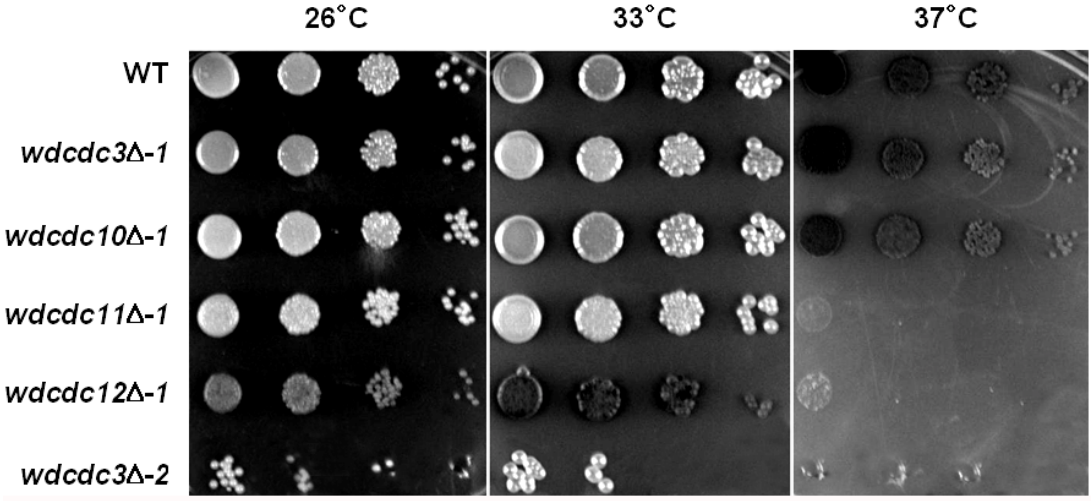
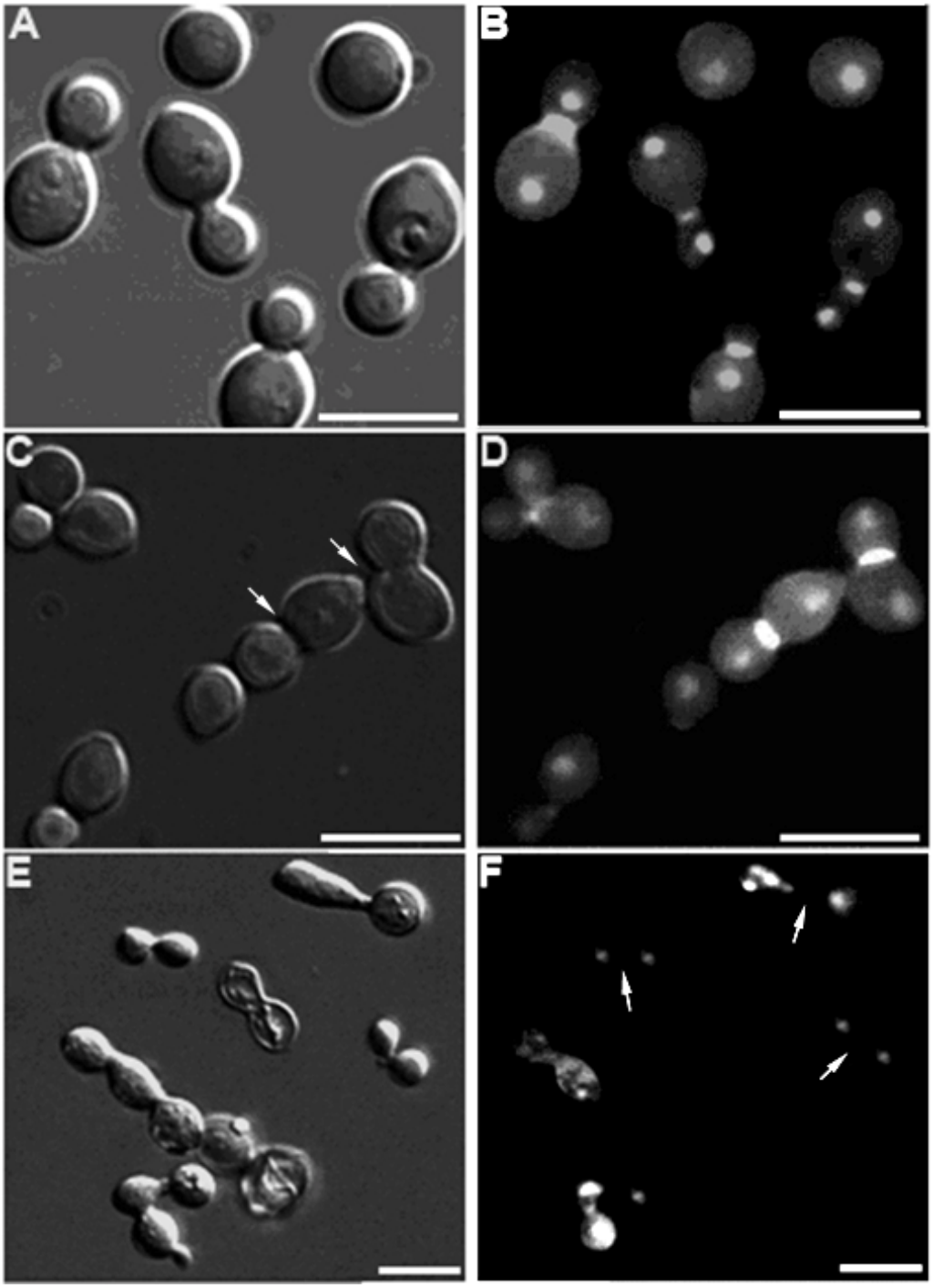

26˚C
33˚C
37˚C
WT
wdcdc3Δ-1
wdcdc10Δ-1
wdcdc11Δ-1
wdcdc12Δ-1
wdcdc3Δ-2

Figure 21. Aberrant morphologies and cytokinesis defects associated with *wdcdc10*Δ-*1* and *wdcdc12*Δ-*1*. (A) WT. (B) Cells in A stained with DAPI and Calcoflour white. (C) *wdcdc10*Δ-*1*. Arrows point to bud necks that are wider then those of WT. (D) Cells in C stained with DAPI and Calcoflour white. (E) *wdcdc12*Δ-*1*. (F) Cells in E stained with DAPI and Calcoflour white. Arrows in F point to areas devoid of chitin in bud necks. In each case, cells (1 x 10^7 cells/ml) grown at 26°C were transferred to pre-warmed (37°C) YPD broth and then sampled and observed microscopically in a time-course manner. The cells shown were sampled after culture for 9 h at 37 °C. Bars equal 10 μm.

Figure 21



vortexing (data not shown). At 37°C, the percentages of abnormal *wdcdc10*Δ-*1* cells with wider bud necks and multiply-budded cells were 15.1 % and 25.2% at 12 h, respectively, whereas less than 1% of the WT exhibited either of those abnormalities (Table 7). These results suggested that *wdcdc10*Δ-*1* is important for maintaining normal bud neck integrity and for allowing proper cytokinesis. Nonetheless, and similar to the results with the *wdcdc3*Δ mutants, the distribution of nuclei in the *wdcdc10*Δ-*1* cells was normal (Figure 21D).

Compared to WT, the *wdcdc12*Δ-*1* deletion mutant strain showed poorer growth, was darker, and secreted more pigment into the agar medium at both 26 and 33°C and grew little, if at all, at 37°C (Figure 20). When cultured in a time course manner in liquid medium at 26 and 37°C and observed microscopically, cells of *wdcdc12*Δ-*1* showed very aberrant budding profiles more like those of *wdcdc3*Δ-*1* than those of *wdcdc10*Δ-*1* (Figure 21E). For example, at 0 h, the WT showed a roughly equal distribution among the three types of cells typical of a culture in early log phase, *i.e.* unbudded cells, cells with a small bud, and cells with a large bud (Table 7). However, about 50 to 60% of the *wdcdc12*Δ-*1* cells either chained or had a large mother cell with at least one abnormally large and elongated bud (Figure 21E). Furthermore, among the cells with large buds (Table 7), 30 to 40% had more than three buds, about 11 to 12% of the cells possessed multiple-nuclei, and roughly 14 to 16% appeared collapsed (Figure 21E and F). These observations suggested that the normal yeast cell cycle progression was disturbed in the *wdcdc12*Δ-*1* strain by loss of polarity and cell separation failure, thereby producing large cells or chained cells or both similar to those of the *wdchs1*Δ*wdchs2*Δ double chitin synthase mutant of *W. dermatitidis* (Zheng *et al*., 2006).

In addition to its inability to grow on agar or in broth media at 37°C (Figure 22A), cells of the insertion disruption mutant, *wdcdc11*Δ-*1*cultured at 26°C were abnormally defective in bud neck width, septum formation, and cell separation, even though they showed no apparent cytoplasmic abnormalities and normal nuclear distribution (Figure 22B). In this respect, the *wdcdc11*Δ-*1* strain showed significant percentages of cell groups consisting of three, four, and even more attached cells (Figure 22B), a phenotype again reminiscent of that of the chitin synthase mutant, *wdchs1*Δ-*1* (Zheng *et al*., 2006). As might be expected, when cells of this mutant were shifted from 26°C to 37°C, they developed even more pronounced cellular defects. For example, after culture for 48 h at 26°C, only about 12% of the *wdcdc11*Δ-*1* cells appeared to be collapsed and most likely dead as judged by tryphan blue staining (Table 8), whereas at 37°C that number increased to 64%. Among the cells appearing dead, some also showed cell wall ruptures similar to those sometimes seen with *wdcdc3*Δ-*2* (Figure 18B g). Nonetheless, in the manner of the *wcdc3*Δ and *wdcdc10*Δ mutants, the *wdcdc11*Δ-*1* mutant showed a normal nuclear distribution (Figure 22B), suggesting that the function of WdCdc11p is to bring about proper cytokinesis, maintain normal septal width, and to sustain viability at high temperature.

3. *WdCDC12* is required for the localized chitin deposition.

To determine whether septins affect the location of chitin deposition during yeast growth, each type of *wdcdc* mutant was stained with Calcoflour white. In the manner of WT (Figure 21B), chitin was mainly deposited in the bud neck of the *wdcdc10*Δ-*1* (Figure 21D), and *wdcdc11*Δ-*1* (Figure 22B) strains at either 26°C or 37°C. However, the

Figure 22. Growth and cytokinesis defects associated with *wdcdc11*Δ-*1* cells showing defects in growth and cytokinesis. (A) Growth profiles obtained in a time course manner from cells (1 x 10^7 cells/ml) cultured in YPD broth at 26°C and 37°C. ■: WT at 26°C, □: *wdcdc11*Δ-*1* at 26°C, ▲: WT at 37°C, Δ: *wdcdc11*Δ-*1* at 37°C. (B) Left: cellular morphologies of *wdcdc11*Δ-*1* cells grown at 26°C; right: same cells stained with DAPI and Calcoflour white.

Figure 22

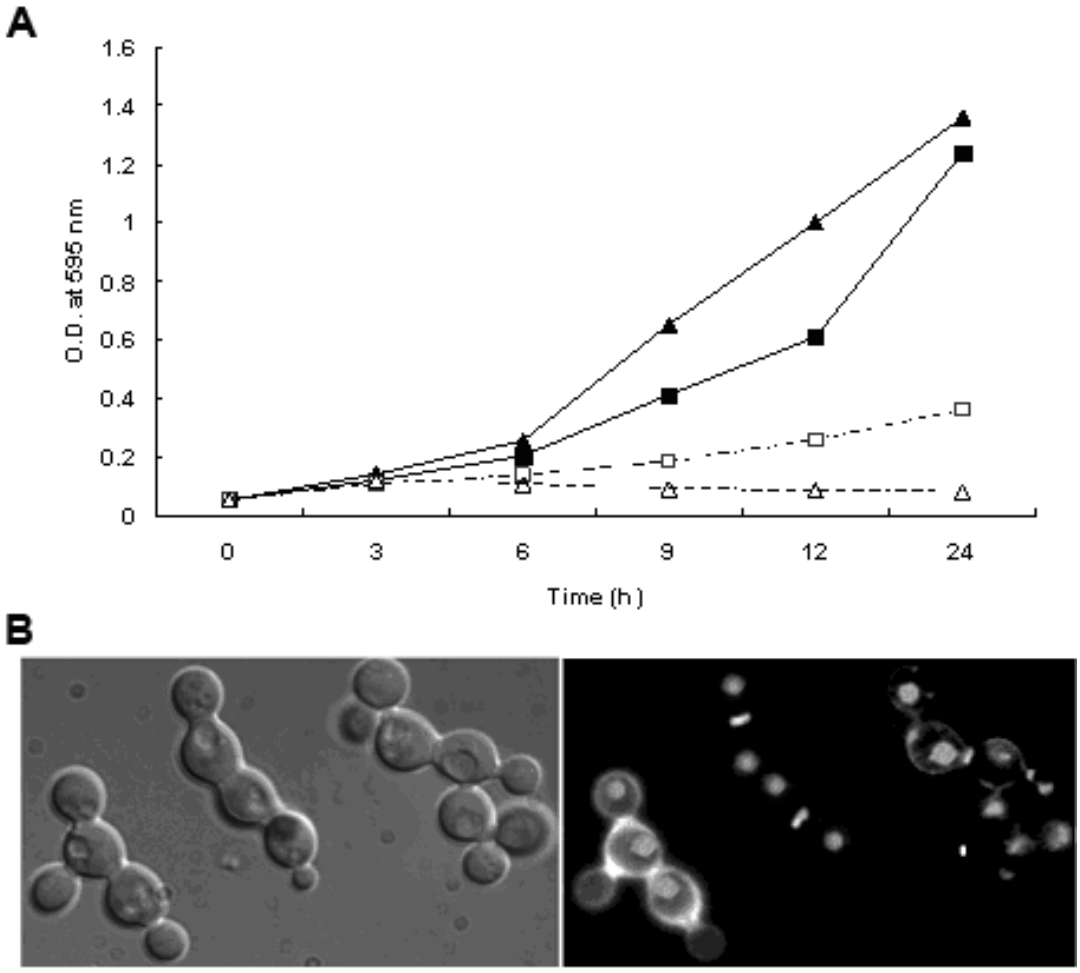


Table 8. Cytokinesis defects of *wdcdc11*Δ*-1*.

Time	Phenotypes of *wdcdc11*Δ*-1* (%)											
	26 ˚C				33 ˚C				37 ˚C			
	Cells w/ three buds	Cells w/ four buds	Cells w/ >4 buds	Dead	Cells w/ three buds	Cells w/ four buds	Cells w/ >4 buds	Dead	Cells w/ three buds	Cells w/ four buds	Cells w/ >4 buds	Dead
0 h	14.2	1.5	0	0	14.2	1.5	0	0	14.2	1.5	0	0
3 h	25.6	7.4	7	0	18.2	14.8	9.8	0	40.6	7.2	8.7	0
6 h	32.6	14.2	5.2	0	27.8	16.8	19.7	0	23.6	20.7	20.1	0
9 h	38.8	12.2	12.7	0	35.3	15.4	19.2	0	27.1	23.5	20.9	1.4
12 h	38.8	9.2	14.4	0	30.9	21.9	26.4	0	24	18.4	24.7	13.3
24 h	35.3	8.6	21.5	0	27.3	18.8	23.6	12.6	23.3	13.9	17.9	31.7
48 h	31.3	7.8	27.3	11.8	24.7	16.9	19.1	23.5	10.9	7.8	6.7	64.2

Values are the averages of the three independent counts of the 100 cellular units.

cells of the *wdcdc3*Δ-*2* mutant strain showed aberrant septa localization (Figure 19). Furthermore, a significant number of the *wdcdc12*Δ-*1* cells did not deposit chitin to their bud necks (Figure 21F). Collectively, these results suggested that at least WdCdc12p is strongly required for chitin deposition in the bud neck, whereas the other septins are required to a lesser degree, if at all.

4. Genetic interactions between *WdCDC10* and *W. dermatitidis* chitin synthase genes.

In *S. cerevisiae*, at the time of bud emergence, Chs3p (a class IV chitin synthase) localizes at the bud neck through an interaction with Chs4p (a Chs3p activation regulator), which in turns associates with Bni4p (a bud neck scaffold protein) that is then finally recruited by Cdc10p to the bud neck (De Marini *et al*., 1997). To determine if a similar interactions exist in *W. dermatitidis*, because bud neck width profile and chitin deposition were often affected when *WdCDC* septin genes were disrupted, attempts were made to introduce a *wdcdc10*Δ-*1* mutation into each of five previously derived strains that had a different chitin synthase gene mutated by insertion or replacement with the *hph* gene (Figure 23A). Other double mutant strains were derived in a manner similar to that used for *wdcdc10*Δ-*1wdchs4*Δ-*1*. After transformation, the double mutants were selected on SD+Hyg+Sur plates and confirmed to have *wdcdc10* deletions by PCR (Figure 23A) and Southern blotting (Figure 24). However, for unknown reasons, no double mutants could be derived in the *wdchs1*Δ background.

In the manner of the WT strain and the *wdcdc10*Δ-*1* and *wdchs2*Δ-*1* single mutant strains, the *wdcdc10*Δ-*1wdchs2*Δ-*1* double mutant strain showed no apparent

Figure 23. Genetic interactions between *W. dermatitidis* septin genes and chitin synthase genes. (A) Left, schematic strategy for the construction; right, PCR confirmation of the *wdcdc10*Δ-*1wdchs4*Δ-*1* double deletion mutant strain. Lane 1: *WdCDC10wdchs4*Δ-*1*, lane 2: *WdCDC10wdchs4*Δ-*1* with an ectopic integration of *wdcdc10*Δ-*1*, lane 3: *wdcdc10*Δ-*1wdchs4*Δ-*1*; Note, the other *wdcdc10*Δ*wdchs*Δ mutants were constructed and evaluated similarly. (B) Growth of the *wdcdc10*Δ*wdchs*Δ double mutants cultivated at 26 and 37°C for 3 days on the YPD agar medium. (C) Ten-fold dilutions of the wild type (WT), *wdchs5*Δ-*11*, and *wdchs5*Δ-*11wdcdc10*Δ-*1* strains spotted on the YPD plates and incubated at 26°C and 37°C for 3 days.

Figure 23

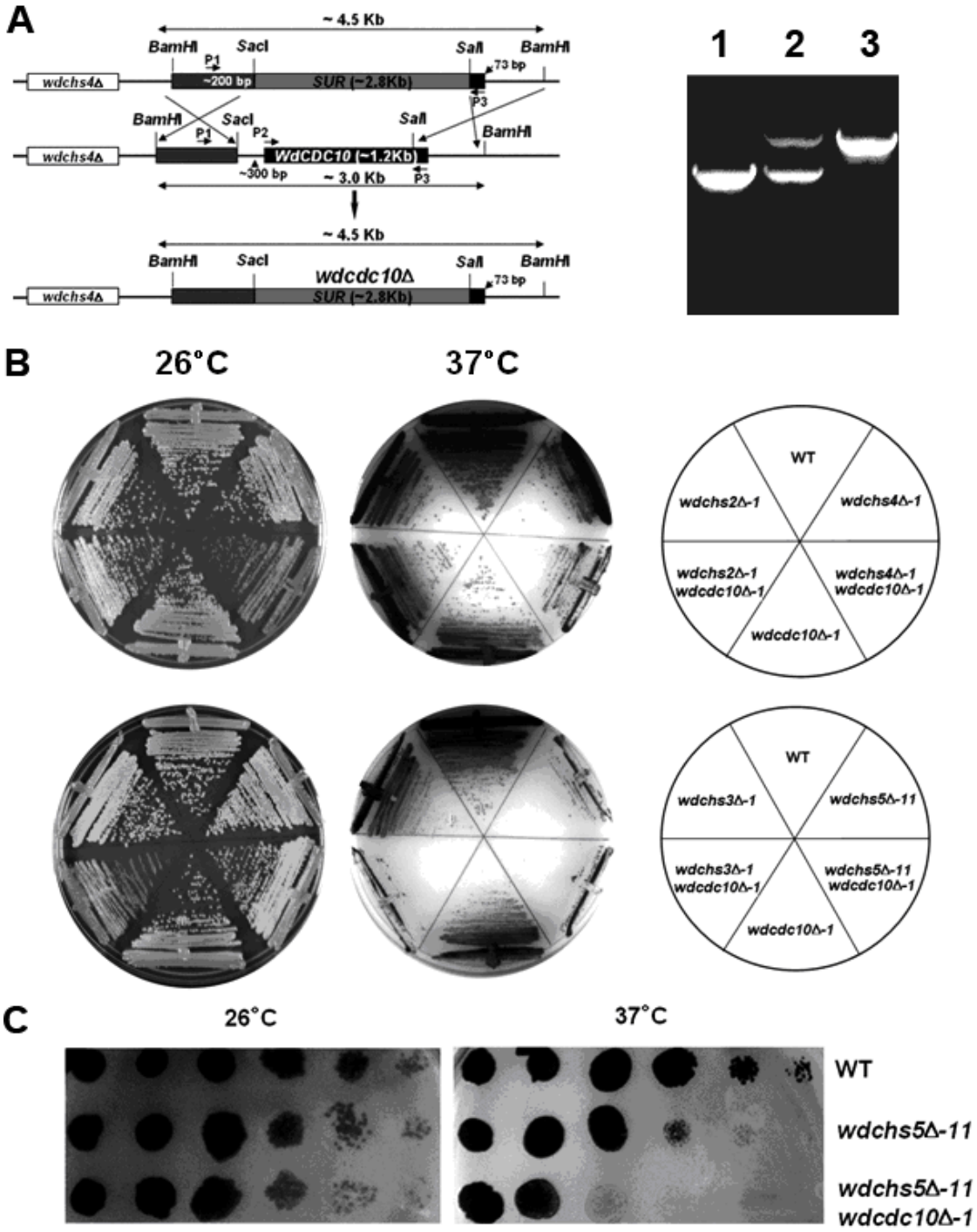
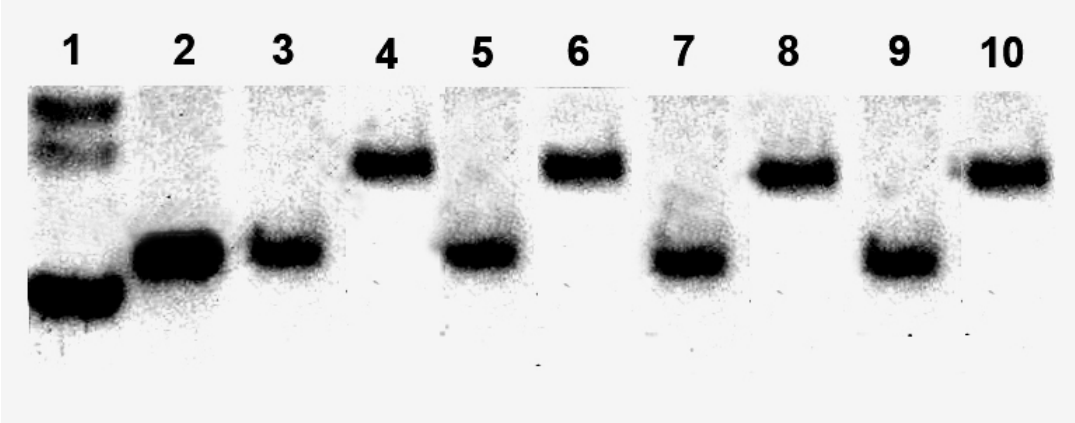

A
~ 4.5 Kb
BamHI
SacI
SalI
BamHI
P1
~200 bp
SUR (~2.8Kb)
73 bp
P3
wdchs4Δ
P2
WdCDC10 (~1.2Kb)
~300 bp
~ 3.0 Kb
wdcdc10Δ
1
2
3
B
26°C
37°C
WT
wdchs2Δ-1
wdchs4Δ-1
wdchs2Δ-1 wdcdc10Δ-1
wdchs4Δ-1 wdcdc10Δ-1
wdcdc10Δ-1
wdchs3Δ-1
wdchs5Δ-11
wdchs3Δ-1 wdcdc10Δ-1
wdchs5Δ-11 wdcdc10Δ-1
C
26°C
37°C
WT
wdchs5Δ-11
wdchs5Δ-11 wdcdc10Δ-1

Figure 24. Southern blot confirmation of the successful derivation of the septin and chitin synthase double deletion mutant strains. Lane 1: 1-kb ladder (NEB). Lane 2: The wild type strain w/ *WdCDC10* probe. Lane 3: The *wdchs2*Δ-*1* strain w/ *WdCDC10* probe. Lane 4: The *wdchs2*Δ-*1wdcdc10*Δ-*1* strain w/ *WdCDC10* probe. Lane 5: The *wdchs3*Δ-*1*strain w/ *WdCDC10* probe. Lane 6: The *wdchs3*Δ-*1wdcdc10*Δ-*1* strain w/ *WdCDC10* probe. Lane 7: The *wdchs4*Δ-*1*strain w/ *WdCDC10* probe. Lane 8: The *wdchs4*Δ-*1wdcdc10*Δ-*1* strain w/ *WdCDC10* probe. Lane 9: The *wdchs5*Δ-*1* strain w/ *WdCDC10* probe. Lane 10: The *wdchs5*Δ-*1wdcdc10*Δ-*1* strain w/ h*WdCDC10* probe.

Figure 24



growth defects (Figure 23B and 25A) or bud neck chitin deposition abnormalities (Zheng *et al*., 2006). Also, the cellular morphology of the *wdcdc10*Δ-*1wdchs2*Δ-*1* double mutant appeared to be identical to that of the *wdcdc10*Δ-*1* single mutant, indicating that no or only negligible genetic interactions existed between *WdCDC10* and *WdCHS2*. Consistent with previous results (Wang *et al*., 1999; Wang and Szaniszlo, 2000), the growth of the *wdchs3*Δ-*1* also appeared normal at both 26°C and 37°C, whereas that of the w*dcdc4*Δ-*1* strain was only somewhat repressed at 37°C (Figure 23B). In contrast, the *wdcdc10*Δ-*1wdchs3*Δ-*1* and *wdcdc10*Δ-*1wdchs4*Δ-*1* double mutants both displayed poorer growth than their *wdchs*Δ single mutant parents at 26°C and 37°C (Figure 23B and 25B). However, the cells of these two mutants did not exhibit enhanced morphological defects (Figure 26 C-F). Nonetheless, because of the poorer growth of the double mutants compared to the WT and the *wdchs*Δ parental strains, I suggest that genetic interactions do exist between *WdCDC10* and *WdCHS3* and *WdCHS4*. However, I concede that it is possible that the two individual mutations involved in each case were such a burden to the cells that they were unable to maintain normal growth rates or chitin deposition patterns.

As previously reported (Liu *et al*., 2004), *wdchs5*Δ-*11* strain showed normal growth at room temperature on YPD agar medium, but showed a prominent ts phenotype at 37°C by 72 h (Figure 23B, C, and 25D). Although, the *wdcdc10*Δ-*1wdchs5*Δ-*11* double mutant also grew well at 26°C (Figure 23C and 25D), at 37°C, this double mutant strain showed even poorer growth than the *wdchs5*Δ-*11* single mutant strain (Figure 23C and 25D). Therefore, the *wdcdc10*Δ-*1wdchs5*Δ-*11* double mutant strain and the *wdchs5*Δ-*11*

Figure 25. Growth of the *wdcdc10*Δ-*1wdchs*Δ double mutant strains. **(**A) ■, WT at 26°C ; □, WT at 37°C; ♦, *wdcdc10*Δ-*1* at 26°C; ◊, *wdcdc10*Δ-*1* at 37°C; ▲, *wdchs2*Δ-*1* at 26°C; Δ, *wdchs2*Δ-*1* at 37°C; ●, *wdcdc10*Δ-*1wdchs2*Δ-*1* at 26°C; ○, *wdcdc10*Δ-*1wdchs2*Δ-*1* at 37°C. (B) ■, WT at 26°C ; □, WT at 37°C; ♦, *wdcdc10*Δ-*1* at 26°C; ◊, *wdcdc10*Δ-*1* at 37°C; ▲, *wdchs3*Δ-*1* at 26°C; Δ, *wdchs3*Δ-*1* at 37°C; ●, *wdcdc10*Δ-*1wdchs3*Δ-*1* at 26°C; ○, *wdcdc10*Δ-*1wdchs3*Δ-*1* at 37°C. (C) ■, WT at 26°C ; □, WT at 37°C; ♦, *wdcdc10*Δ-*1* at 26°C; ◊, *wdcdc10*Δ-*1* at 37°C; ▲, *wdchs4*Δ-*1* at 26°C; Δ, *wdchs4*Δ-*1* at 37°C; ●, *wdcdc10*Δ-*1wdchs4*Δ-*1* at 26°C; ○, *wdcdc10*Δ-*1wdchs4*Δ-*1* at 37°C. (D) ■, WT at 26°C ; □, WT at 37°C; ♦, *wdcdc10*Δ-*1* at 26°C; ◊, *wdcdc10*Δ-*1* at 37°C; ▲, *wdchs5*Δ-*1* at 26°C; Δ, *wdchs5*Δ-*1* at 37°C; ●, *wdcdc10*Δ-*1wdchs5*Δ-*1* at 26°C; ○, *wdcdc10*Δ-*1wdchs5*Δ-*1* at 37°C. Cells ($1x10^7$ cells/ml) grown at 26°C and 37°C YPD broth and sampled in a time course manner. O.D. measured at 595 nm.

Figure 25

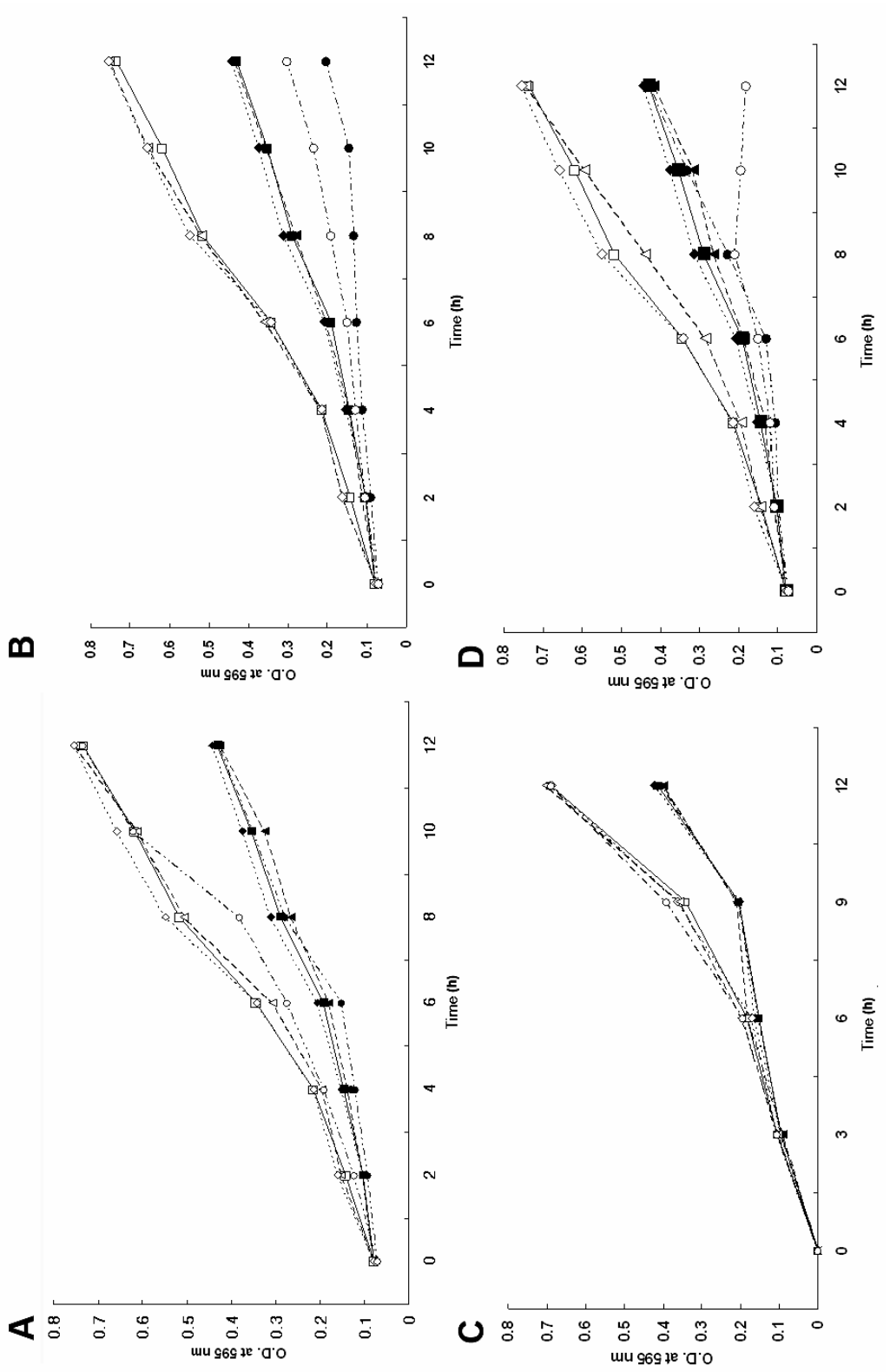
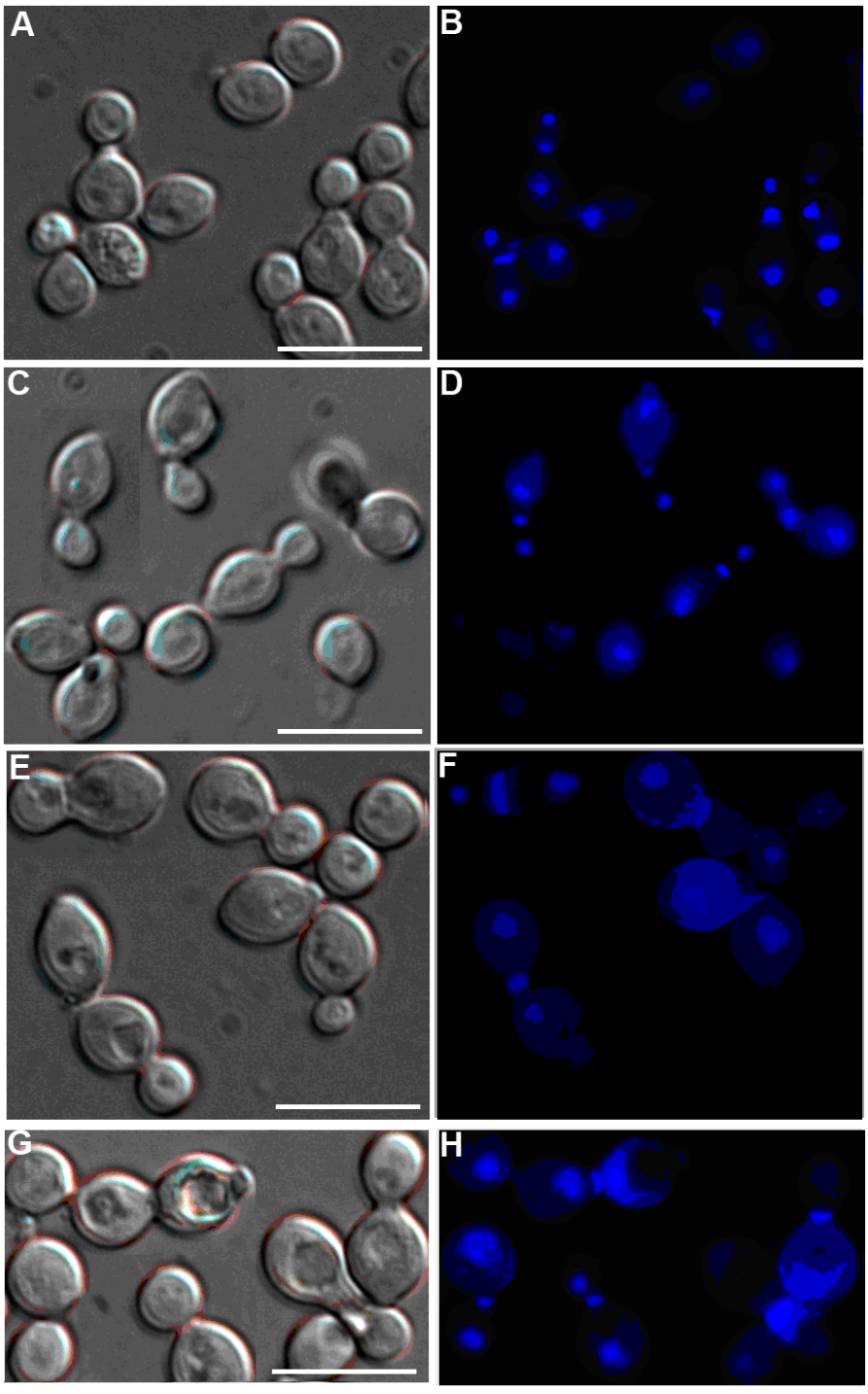


Figure 26. Phenotypes in cells of the *wdcdc10*Δ-*1wdchs*Δ double mutants. Strains were cultured at 37°C (A-F) or at 26°C (G and H) for 9 h. **(**A) *wdcdc10*Δ-*1wdchs2*Δ-*1*. (B) Cells in A stained with DAPI and Calcoflour white. (C) *wdcdc10*Δ-*1wdchs3*Δ-*1*. (D) Cells in C stained with DAPI and Calcoflour white. (E) *wdcdc10*Δ-*1wdchs4*Δ-*1*. (F) Cells in E stained with DAPI and Calcoflour white. (G) *wdcdc10*Δ-*1wdchs5*Δ-*11*. (H) Cells in G stained with DAPI and Calcoflour white. Cells ($1x10^7$ cells/ml) grown at 26°C were transferred into the pre-warmed 37°C YPD broth and sampled in a time course manner. Bars equal 10 μm.

Figure 26

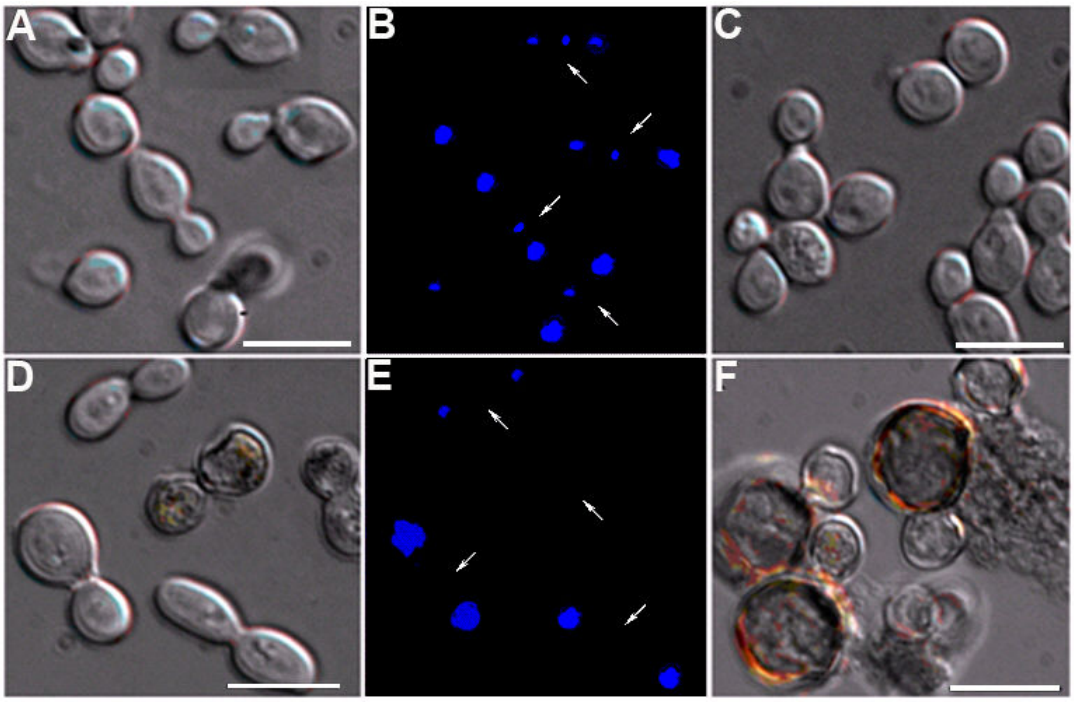


single mutant strain were cultured in a time course manner, sampled every three hours, and compared with respect to the timing of the appearance of the ts phenotype at 37°C: in contrast to that of the *wdchs5*Δ-*11* single mutant, the ts phenotype of the *wdcdc10*Δ-*1wdchs5*Δ-*11* double mutant appeared almost immediately after it was shifted from 26°C to 37°C. Also, many cells of this double mutant had cell diameters that were larger than those of the *wdchs5*Δ-*11* strain (Figure 27F) and showed a bursting balloon-like phenotype, indicating defects in the cell wall integrity. This result suggested that an introduction of the *wdcdc10*Δ-*1* mutation enhanced and accelerated the expression of the ts and cell wall integrity defects of the *wdchs5*Δ-*11* strain.

As reported previously, the *wdchs5*Δ-*11* strain shows uneven chitin deposition at yeast septal regions after prolonged culture (>48 h) at 37°C (Liu *et al*., 2004). Therefore, chitin deposition in the yeast bud neck of the *wdchs5*Δ-*11* (Figure 23B), *wdcdc10*Δ-*1wdchs5*Δ-*11* (Figure 27E), and other *wdcdc*Δ*wdchs*Δ mutants (Figure 26 A-F) were monitored by Calcoflour white staining. At 26°C, chitin was successfully deposited at the bud neck in all the strains including the *wdcdc10*Δ-*1wdchs5*Δ-*11* strain (Figure 26 G and H). While the *wdchs5*Δ-*11* cells also successfully deposited chitin to the yeast bud necks at 37°C at 9 h (Figure 27B), almost all cells of the *wdcdc10*Δ-*1wdchs5*Δ-*11* double mutant strain incubated identically were devoid of such chitin deposits (Figure 27E). This indicated that WdChs5p may have had an interaction with at least WdCdc10p of the septin complex.

Figure 27. Accelerated production of the terminal *wdchs5*Δ-*11* mutant phenotypes in cells of the *wdcdc10*Δ-*1wdchs5*Δ-*11* double mutants. Strains were cultured at 37°C for 9 h (A and D) or 12 h (C and F). **(**A) *wdchs5*Δ-*11*. (B) Cells in A stained with DAPI and Calcoflour white. (C) *wdchs5*Δ-*11*. (D) *wdcdc10*Δ-*1wdchs5*Δ-*1*. (E) Cells in D stained with DAPI and Calcoflour white. (E) *wdcdc10*Δ-*wdchs5*Δ-*111*. Cells ($1x10^7$ cells/ml) grown at 26°C were transferred into the pre-warmed 37°C YPD broth and sampled in a time course manner. Arrows in B indicate bud beck chitin deposition, whereas in E they indicate the absence of chitin at the bud necks. Bars equal 10 μm.

Figure 27



5. Septins are required for the proper localization of WdChs5p during budding yeast growth.

In *S. cerevisiae*, Bni1p is required for the formation actin cables and motors, such as class V myosins, and use this array to transport secretory vesicles (Evangelista *et al*., 2003). Mutants with a *BNI1* deletion assemble a septin ring with a wider diameter than that of wild-type cells (Kadota *et al*., 2004), which is consistent with our findings of wider bud necks among some cells of *wdcdc*Δ septin mutants, such as *wdcdc10*Δ-*1*. Previously, it was suggested that the myosin motor-like domain of the WdChs5p of *W. dermatitidis* possibly transports WdChs5p using an actin cable-based system (Liu *et al*., 2004). Therefore, I asked whether WdChs5p requires the septin complex for its cellular localization during budding yeast growth. To answer this question, we produced anti-WdChs5pMyo12 rabbit antibodies (polyclonal, 1:100) that specifically recognize the end region of the myosin-motor like domain (Myo12) of WdChs5p. As expected the expression of WdChs5p was not detected in cells of the *wdchs5*Δ-*11* mutant and, consistent with the previous report (Liu *et al*., 2004), was lower in cells cultured at 26°C than in those cultured at 37°C (Figure 28). After the antibody was conjugated with anti-rabbit goat antibody FITC (1:250, Sigma), it revealed that WdChs5p localized to yeast mother cells, daughter buds, and yeast bud neck regions at 37°C in the WT strain. In this respect, as the daughter cells developed, WdChs5p first localized at the tip of a bud, next spread along the cell wall as the bud enlarged, and finally also localized at the bud neck (Figure 29C), indicating that WdChs5p localizes to sites of the polarization. However, little or no signal was detected when the WT was cultured at 26°C, probably because of the low expression level of WdChs5p (Figure 28), or in *wdchs5*Δ-*11* cultured identically

Figure 28. The anti-WdChs5pMyo12 rabbit antisera recognized WdChs5p specifically. Immunoblotting of membrane proteins isolated from the wild-type (WT) and *wdchs5*Δ*-11* cultured at 26^{o}C and 37^{o}C. Lane 1, WT membrane proteins from 37°C cells; lane 2, WT membrane proteins from 26°C cells; lane 3, *wdchs5*Δ*-11* membrane proteins from 37°C cells. Membrane proteins were first solubilized in 1% digitonin buffer, then resolved in a 10% SDS PAGE gel, and finally transferred for the immunoblots to a nitrocellulose membrane. The membrane was subsequently incubated in the following buffers: TBST (0.1% Tween 20 in TBS, pH 7.4) with 5% non-fat dry milk, next in TBST with primary anti-Myo12 antibody diluted 1:5,000, and finally in TBST with 1:10,000 of horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Zymed). Bound antibody was detected with an enhanced chemiluminescence system (Amersham Pharmacia) according to the manufacturer's protocol (courtesy of Dr. Dariusz Abramczyk, The University of Texas at Austin)..

Figure 28

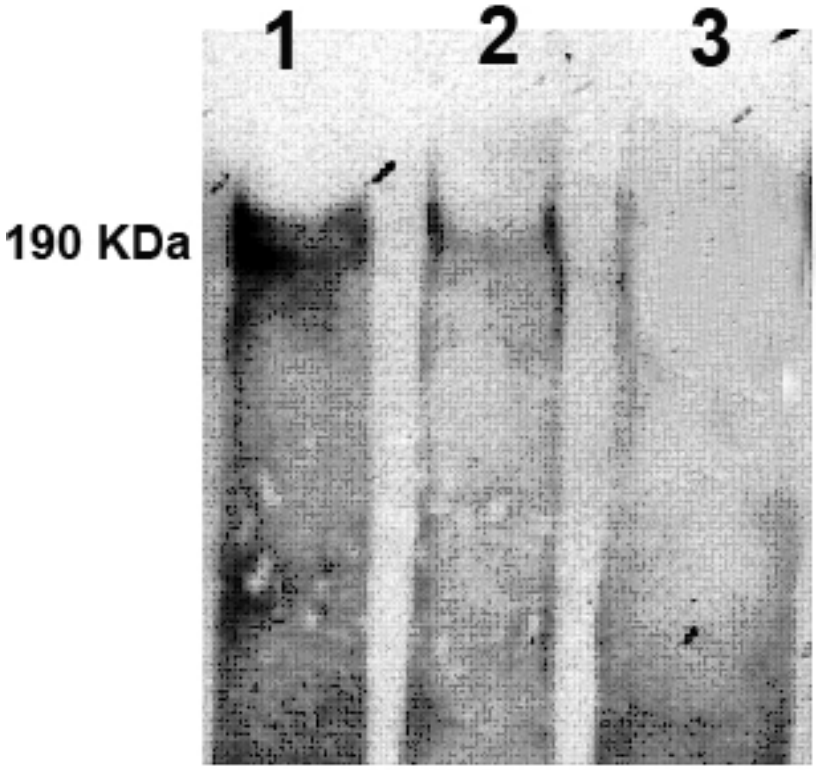
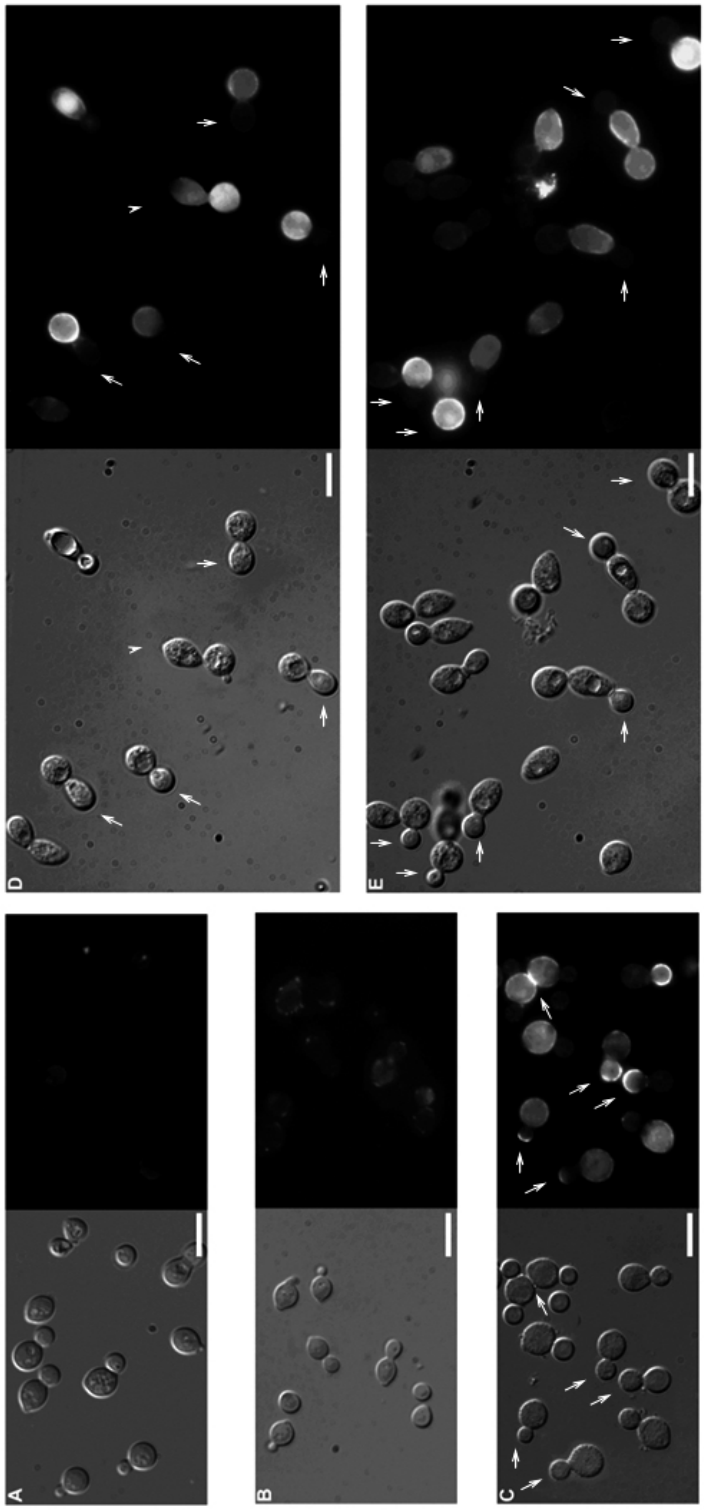


Figure 29. WdChs5p localization during yeast budding growth in the *W. dermatitidis*. **(**A) *wdchs5*Δ*-11* cultured at 37°C for 24 h, note absence of a FITC signal for WdChs5p in panel at right (B) WT without anti-WdChs5p rabbit antibody cultured at 37°C for 24 h, note absence of a FITC signal for WdChs5p in panel at right (C) WT cultured at 37°C for 24 h, note arrows point to sites on cells where FITC signals were detected in the enlarging buds and at septal regions. (D) *wdcdc3*Δ*-1* cultured at 37°C for 24 h, note arrows point to enlarging buds with no FITC signals (E) *wdcdc10*Δ*-1* cultured at 37°C for 24 h, note arrows point to enlarging buds with no FITC signals detected in all cases. Cells ($1x10^7$ cells/ml) for inoculum were first grown at 26°C and then transferred into the pre-warmed 37°C YPD broth and sampled after 24 h. After formaldehyde (final 5% conc.) fixation for 2 h, cells were washed with 1X PBS. Spheroplasts were prepared by incubation of yeast cells with zymolyase (1 μg/ml, ZYMO Research, USA) in spheroplast buffer [0.9 M sorbitol, 0.1 M EDTA, 1% (V/V) beta-mercaptoethanol] for 1 h at 30°C and then incubated with anti-WdChs5p Myo12 rabbit antisera (1:100) in 1X PBS buffer containing 1 mg/ml BSA and 0.03% (V/V) NaN_3 for 2 h, followed by washing three times with 1X PBS buffer. After incubating with the FITC-conjugated anti-rabbit goat antibody (1:250, Sigma, USA) in 1X PBS buffer containing 1 mg/ml BSA and 0.03% (V/V) NaN_3 for 1 h, the cells were finally examined and photographed. Bars equal 10 μm.

Figure 29



(Figure 29A). In contrast to the WT strain grown at 37°C, signals were also not observed in the daughter cells of the *wdcdc10*Δ*-1* strain (Figure 29E), although weak signals were detected in mother cells and bud necks (Figure 29E). Interestingly, the distribution of the WdChs5p FITC signals in the *wdcdc3*Δ*-1* strain (Figure 29D) was identical with those of that of the *wdcdc10*Δ*-1* strain. Taken together, these results suggest that WdChs5p localizes at sites of polarized cell wall growth in daughter cells and requires an intact septin complex.

To determine whether WdChs5p uses microtubules or actins for transport, WT cells were cultured at 37°C in YPD broth containing nocodazole (20 µg/ml), a known inhibitor of microtubule polymerization in *W. dermatitidis* (Jacobs and Szaniszlo, 1982), *S. cerevisiae* (Holm *et al*., 1985; Pillus and Solomon, 1986), and other fungi (Walker, 1982; Yokoyama *et al*., 1990) Consistent with previous results that showed mitosis, but not bud formation, were inhibited in *W. dermatitidis* (Jacobs and Szaniszlo, 1982) incubated with nocodazole, and regardless of the number of buds associated with a mother cell, WT yeast showed only one nucleus in the presence of nocodazole (Figure 30 b, e, and h). Nonetheless, WdChs5p still localized to the daughter cells even in the absence of mitosis and nuclear migration (Figure 30 c, f, and i), indicating that microtubules are not required for the normal localization of WdChs5p.

Because, for unknown reasons, we have had no success over the years in staining the actins of *W. dermatitidis*, WT cells were cultured in YPD broth containing latrunculin A (LAT-A, final conc. 200 µM), an actin polymerization inhibitor (Ayscough *et al*., 1997) to determine whether WdChs5p transport is associated with actin. As shown in

Figure 30. WdChs5p localization in budding WT yeast cells inhibited in microtubule function. (a-i) The mitotic cycle was arrested (note presence of only one nucleus) in cells with cultured nocodazole (20 ug/ml) for 8 h culture. (j-o) 135 min after removing the nocodazole from the medium cells are released from the mitotic cycle block and now have more than one nucleus. In each case, cells ($1x10^7$ cells/ml) grown at 26°C were transferred into pre-warmed 37°C YPD broth, sampled after 24 h. After staining with the FITC-conjugated anti-rabbit goat antibody, nuclei were also stained with 1X PBS buffer containing 5 µg/ml DAPI, for 10 min, followed by washing with 1X PBS. Bars equal 10 µm.

Figure 30

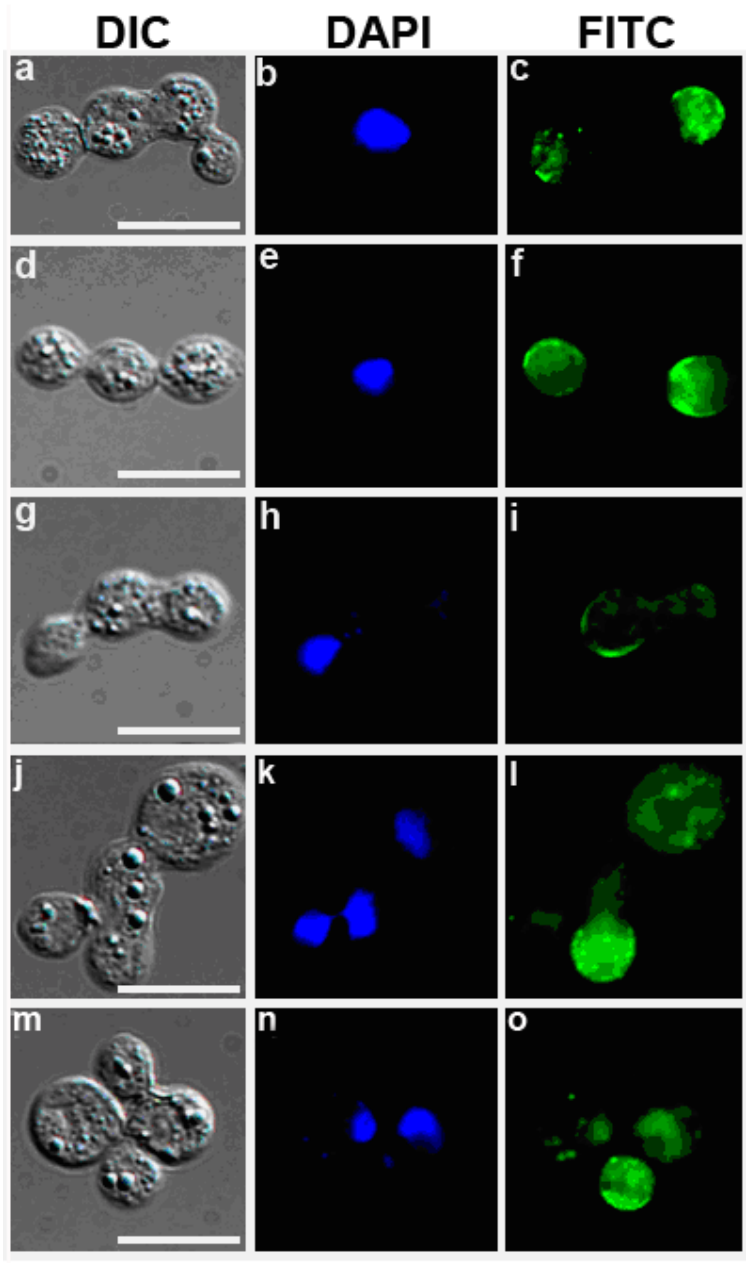


Figure 31, the bud tip localization of WdChs5p was affected in the presence of latrunculin. In the WT cells, as daughter cells developed, WdChs5p first localized at the tip of a bud, next spread from that point toward the mother cell as the bud enlarged, and finally also localized at the bud neck (Figure 29C). In contrast, in the presence of latrunculin, the signals for WdChs5p were unable to be observed in the bud tips of most cells (Figure 31 C, F, and J). In addition, WdChs5p aberrantly localized in the bud neck (Figure 31 C and G) compared to that of the cells in the absence of latrunculin (Figure 29C). Interestingly, when the cells were exposed to latrunculin longer than 30 min, no signal for WdChs5p was observed (Figure 31L and P). Collectively, these strongly suggest that the normal localization of WdChs5p requires actin. This strongly suggests that *W. dermatitidis* use actin based transport system for WdChs5p localization in the yeast budding cells.

6. *WdCDC3* and *WdCDC11* are required for the conidiogenesis.

Septins and chitin synthases are important for sporulation in fungi. Therefore, to understand the functions of the *W. dermatitidis* septins and chitin synthases in hyphal development and asexual sporulation, the *W. dermatitidis* strains were incubated in a thin layer of PDA on slides and cultured at 26ºC for 20 days to induce mycelium and conidium development for microscopic observation. Under our experimental conditions, numerous chitin synthase mutants (Figure 32, F-J), including the *wdcdc10*Δ*-1wdchs*Δ double mutants (Figure 32, K-M), displayed no defects in hyphal development or conidiogenesis. Similarly, neither the *wdcdc10*Δ*-1* (Figure 32D) nor the *wdcdc12*Δ*-1*

Figure 31. WdChs5p localization in budding WT yeast cells inhibited in actin function. Cells were exposed to latrunculin A (LAT-A, final conc. 200 μM) for 20 min (A-I) and for 30 min (J-P) after shift from 26°C to 37°C. Arrows indicate absence of WdChs5p at the growing bud tips. Cells ($1x10^7$ cells/ml) grown at 26°C were transferred into the pre-warmed 37°C YPD broth and sampled after 24 h and then treated as above except that after staining with the FITC-conjugated anti-rabbit goat antibody, nuclei were also stained with 1X PBS buffer containing 5 μg/ml DAPI, for 10 min, followed by washing with 1X PBS. Arrows indicate aberrant localization of WdChs5p. Bars equal 10 μm.

Figure 31

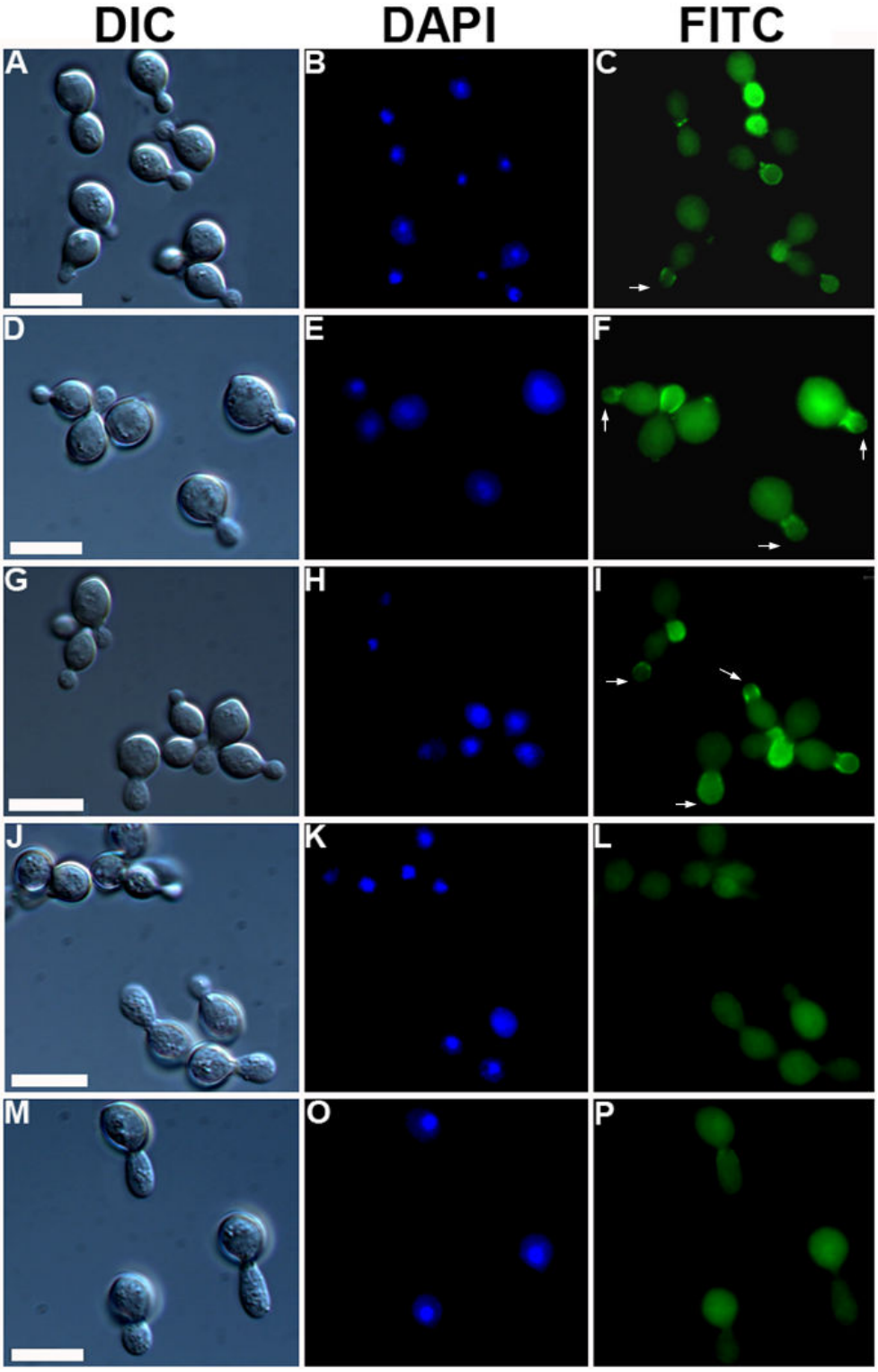
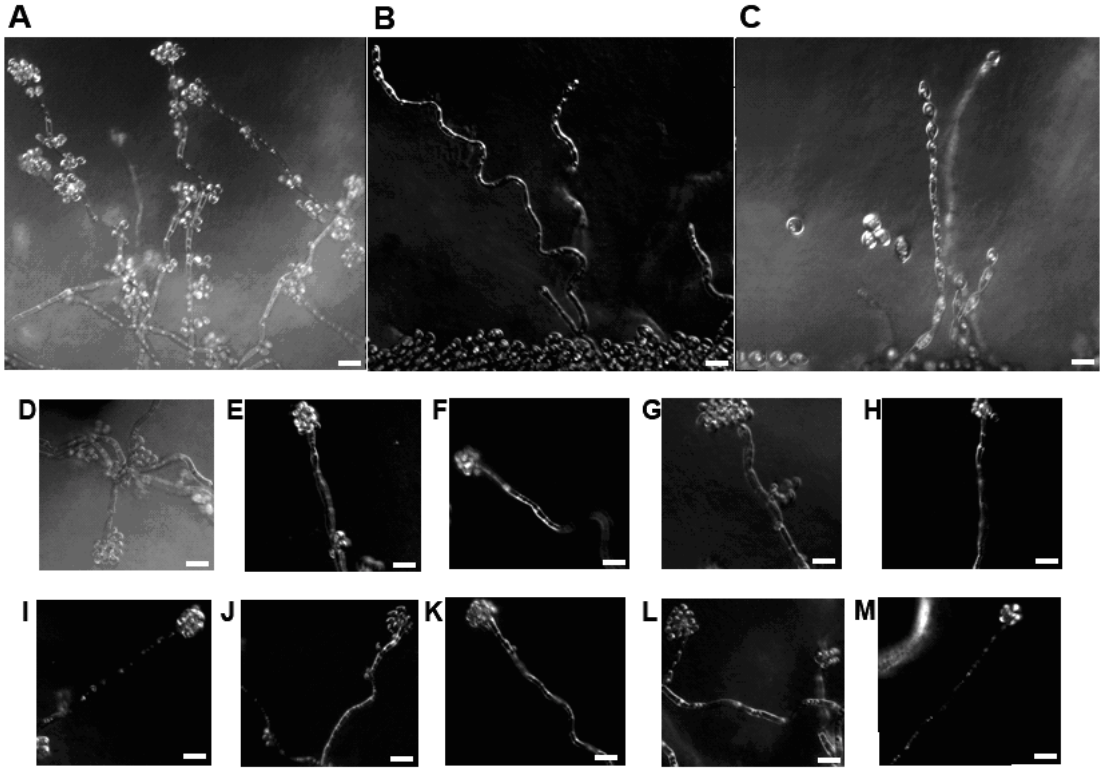


Figure 32. Conidial and hyphae development by the *W. dermatitidis* strains. Cells were cultured on a slide on a thin layer of Potato Dextrose Agar (PDA) at 26°C for 20 days. A: wild type, B: *wdcdc3*Δ*-1*, C: *wdcdc11*Δ*-1*, D: *wdcdc10*Δ*-1*, E: *wdcdc12*Δ*-1*, F: *wdchs1*Δ*-1*, G: *wdchs2*Δ*-1*, H: *wdchs3*Δ*-1*, I: *wdchs4*Δ*-1*, J: *wdchs5*Δ*-11*, K: *wdchs2*Δ*-1wdcdc10*Δ*-1*, L: *wdchs3*Δ*-1wdcdc10*Δ*-1*, M: *wdchs5*Δ*-11wdcdc10*Δ*-1*. Note absence of conidiogenesis in B and C, Bars equal 10 μm.

Figure 32



(Figure 32E) strains showed defects in either hyphal development or conidiogenesis. However, no conidia were produced by the *wdcdc3*Δ*-1* (Figure 32B) and *wdcdc11*Δ*-1* (Figure 32C) strains, even though both septin mutant strains had no defect in hyphal development. I suggest that *WdCDC3* and *WdCDC11* are required for conidium development in *W. dermatitidis*, possibly because it entails a budding mechanism (Oujezdsky and Szaniszlo, 1974).

IV. DISCUSSION

Originally identified in *S. cerevisiae* by their functions in cytokinesis, septins subsequently were found to play a template role in recruiting various other proteins to the yeast bud neck and to have even broader roles in the cellular development of other fungi and higher eukaryotes. In this study, I characterized the functional roles played by four vegetative septins in *W. dermatitidis,* which is a model system for studying fungal development in a conidiogenous mold capable of yeast growth (Szaniszlo, 2006). Analysis of four septin genes (*WdCDC3*, *WdCDC10*, *WdCDC11*, and *WdCDC12*) cloned from *W. dermatitidis* revealed that their deduced protein products had significant homologies to septin orthologs of other fungi (Table 5). Consistent with findings about other fungal septins (Versele *et al*., 2004; Casamayor and Synder, 2002), each *W. dermatitidis* septin had all of the expected conserved domains: the N-terminus polybasic resides, a GTPase domain, and a C-terminus coiled coil domain, except in WdCdc10p (Figure 13). Interestingly, and in spite of its relatively low homology with *CDC3* of *S. cerevisiae*, the *WdCDC3* cDNA completely rescued the *cdc3-6* mutant phenotypes of that fungus at the restrictive temperature (Figure 14). While *WdCDC12* also complemented the corresponding *S. cerevisiae* ts mutant partially (Table 6), cells still elongated (data not shown), a result consistent with that obtained with *AspC*, which complements partially the *S. cerevisiae cdc3* and *cdc12* ts mutants (personal communication, Dr. M. Momany, University of Georgia at Athens). These two results suggested that at least *CDC3* and *CDC12* and *WdCDC3* and *WdCDC12* respectively, are conserved not only in structure, but also in function among some species.

A. Functional properties of *W. dermatitidis* septins during vegetative growth.

Two interesting differences were identified by analysis of septin function by mutagenesis in *W. dermatitidis*, compared to results reported with other fungi. First, whereas *CDC3* homologs are essential genes in *S. cerevisiae* (Longtine *et al*., 2000), *C. albicans* (Warenda and Konopka, 2002), and *A. nidulans* (*aspB*, Momany *et al*., 2001), *WdCDC3* is not essential in *W. dermatitidis*. However, it is critical for maintaining viability, since *wdcdc3*Δ*-2* cells grew poorly and many eventually died under our experimental conditions (Figure 18A). Second, unlike other *CDC11* homologs in fungi (Longtine *et al*., 2000; Warenda and Konopka, 2002; Momany *et al*., 2001; An *et al*., 2004), *WdCDC11* seems essential, suggesting that WdCdc11p has a unique and important function in *W. dermatitidis*. In contrast to these differences, and consistent with findings about their homologs in other fungi, *WdCDC10* is a nonessential gene, whereas *WdCDC12* seems to be an essential gene. Furthermore, *wdcdc10*Δ*-1* cells displayed abnormal morphologies, including wide yeast bud necks and chained cells, at any temperature in the manner of *cdc10* mutants of *S. cerevisiae* (Flesher *et al*., 1993), a finding that contrasts with that of *C. albicans cdc10*Δ cells, which are phenotypically wild type at 30°C or below (Warenda and Konopka, 2002). Unlike *cdc10*Δ cells of *S. cerevisiae*, however, cells of both *C. albicans cdc10*Δ (Warenda and Konopka, 2002) and *wdcdc10*Δ*-1* (Figure 20) remained viable and were not strongly affected in reproductive growth at elevated temperatures.

In agreement with results with *cdc11*Δ and *cdc12*Δ mutants of *S. cerevisiae* and *C. albicans* (Hartwell, 1971; Warenda and Konopka, 2002), the *wdcdc11*Δ*-1* and

*wdcdc12*Δ*-1* cells lost viability at 37°C with prolonged culture (Figure 20). Furthermore, these particular *wdcdc* mutants also commonly displayed similar cytokinesis defects: e. g. chained cells, aberrant morphologies including wide septal regions, and irregular chitin staining patterns (Figure 19, 21D and F, and 22B), which were most severe in *wdcdc12*Δ*-1* (Figure 21F). In *W. dermatitidis*, chitin is deposited throughout the cell wall during polymorphic vegetative growth, but septal regions are the primary sites of chitin deposition in yeast cells (Cooper *et al*., 1984; Jacobs *et al*., 1985; Harris and Szaniszlo, 1986; Szaniszlo and Momany, 1993). Therefore, I suggest that some chitin synthases responsible for bud neck chitin deposition are not recruited properly by the septin complex in certain of our *wdcdc* septin mutants. Support for septin and chitin synthase interactions is well documented with *S. cerevisiae*. For example, in yeast an interaction between Cdc10p and Bni4p (a bud neck scaffold protein) is necessary for the bud neck recruitment of chitin synthase Chs3p with Chs4p (Chs3p activity cofactor), which also binds to Cdc10p (De Marini *et al*., 1997).

B. Detection of genetic interactions between *WdCDC10* and three *WdCHS* genes.

In previous reports members of the Szaniszlo laboratory described results about the cloning and the effects of disruption of five chitin synthase genes: *WdCHS1* (Zheng *et al*., 2006), *WdCHS2* (Wang *et al*., 2001), *WdCHS3* (Wang and Szaniszlo, 2000), *WdCHS4* (Wang *et al*., 1999), and *WdCHS5* (Liu *et al*., 2004). These studies showed that each *WdCHS* gene encodes a chitin synthase of a different class: WdChs1p (class II), WdChs2p (class I), WdChs3p (class III), WdChs4p (class IV), and WdChs5p (class V). They further showed that isozymes WdChs1p, WdChs2p, and WdChs4p are orthologs of

Chs2p, Chs1p, and Chs3p in *S. cerevisiae*, whereas WdChs3p and WdChs5p have no orthologs in that fungus. By introducing the *wdcdc10*Δ*-1* mutation into four of five different chitin synthase mutant strains (Figure 23B), I now report that genetic interactions have been detected between *WdCDC10* and *WdCHS3*, *WdCHS4*, and *WdCHS5*, but not between *WdCDC10* and *WdCHS2*. While it is unclear why the *wdcdc10*Δ*wdchs1*Δ double mutant could not be derived, it is not surprising that no interactions were detected between *WdCDC10* and *WdCHS2*. This is because the importance of class I chitin synthases (class I) is reported to be minor in fungi (Cabib *et al*., 1992; Gold and Kronstad, 1994; Fujiwara *et al*., 2000; Wang *et al*., 2002). In this respect, WdChs2p (Class I), as in the manner of Chs1p (class I) of *S. cerevisiae*, is believed to be a back up enzyme for WdChs1p (class II), which is thought to be the enzyme most responsible for primary septum formation in *W. dermatitidis* (Wang *et al*., 2002; Zheng *et al*., 2006).

Although the interactions between *WdCDC10* and *WdCHS3*, *WdCHS4*, and *WdCHS5* were not predictable, their detection suggests the followings. First, WdChs3p, which has no ortholog in *S. cerevisiae*, and WdCdc10p together have a novel function in the septin complex that is required for normal *W. dermatitidis* growth (Wang and Szaniszlo, 2000; Roncero, 2002). This hypothesis is based on the finding that although the *wdchs3*Δ*-1* strain showed no dramatic morphological or cytological defects at any temperature (Wang and Szaniszlo, 2000; Wang *et al*., 2002), cells of the *wdcdc10*Δ*-1wdchs3*Δ*-1* strain grew poorer than the WT or the *wdchs3*Δ*-1* and *wdcdc10*Δ*-1* single mutant strains at the temperatures tested (Figure 23B). Second, WdChs4p is recruited or

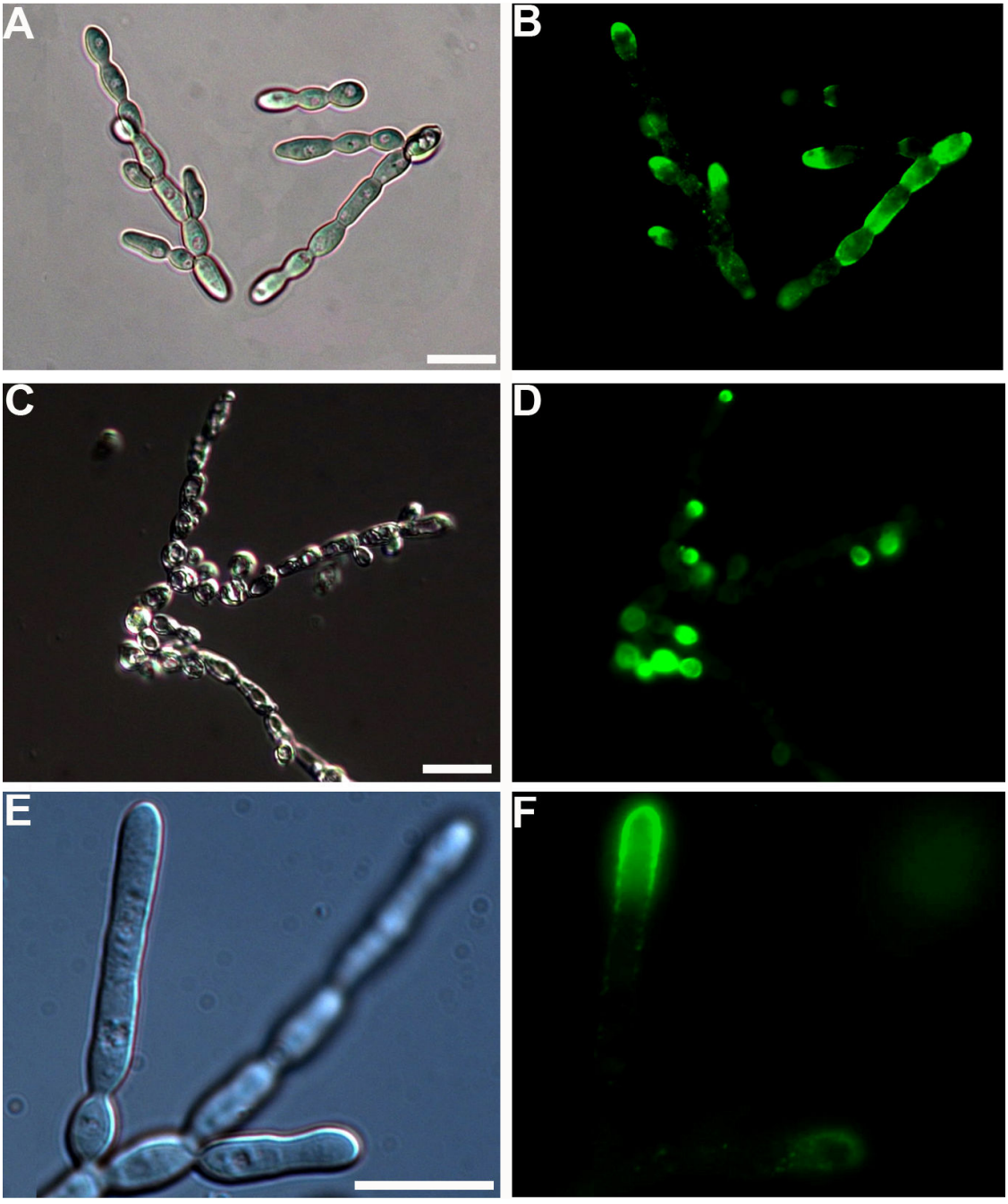
functions in a manner different from that of its Chs3p orthologue in *S. cerevisiae* because only a weak interaction was detected between *WdCHS4* and *WdCDC10*. This was unexpected because in *S. cerevisiae* Chs3p is directly responsible for the chitin ring formation in the bud neck for cytokinesis (De Marini *et al*., 1997). I suspect this difference relates to the fact that chitin rings have never been detected during budding in *W. dermatitidis* (Cooper *et al*., 1984; Jacobs *et al*., 1985). Nonetheless, *wdchs4*Δ*-1* cells do display defects in cell separation (Wang *et al*., 1999) in the manner to that of *S. cerevisiae chs3* (Valdivieso *et al*., 1991), and *C. albicans chs3* (Bulawa *et al*., 1995) mutants, and are reduced in chitin content at 37°C (Wang *et al*., 1999), as are *Neurospora crassa chs4* (Din *et al*., 1996), *A. nidulans chsE*Δ (Specht *et al*., 1996), and *Ustilago maydis chs5* (Xoconostle-Cazares *et al*., 1997). Thus, I expected that WdChs4p would be recruited to the bud neck by a septin based hierarchy protein interaction as in a similar manner of *S. cerevisiae* Chs3p (De Marini *et al*., 1997). Third, the septin complex is required for the bud neck localization and function of WdChs5p in *W. dermatitidis*, because a strong and interesting interaction was detected between *WdCDC10* and *WdCHS5*. Support for this last suggestion was our finding that the introduction of the *wdcdc10*Δ*-1* mutation into the *wdchs5*Δ*-11* strain dramatically enhanced and accelerated its ts growth, morphological, and cytological defects (Figure 27E and F) yet still had no detectable effect at 26°C. Furthermore, when shifted to 37°C, most *wdcdc10*Δ*-1wdchs5*Δ*-11* cells did not deposit chitin at the bud neck (Figure 27E).

C. Septins are required for WdChs5p localization during yeast budding.

Class V chitin synthases are believed to exist only among Ascomycota species that produce conidia. They consist of about 1,500 amino acids and are distinguished by the presence of a myosin motor-like domain in their N-terminus. Knowledge about the functions of the class V enzymes is particularly important to *W. dermatitidis*, because its class V isozyme, WdChs5p, is the only single chitin synthase of this pathogen required to maintain viability at 37°C, the temperature of human infection (Liu *et al*., 2004). By using the anti-WdChs5pMyo12 rabbit antibody I now provide evidence that the septin complex is also required for the normal localization of WdChs5p during yeast budding at 37°C. Two findings support this contention. First, in WT cells, WdChs5p was localized to and was responsible for the cell wall chitin deposition at polarized sites in yeast cultured at elevated temperature (Figure 29C): although not included in my results I have also determined that WdChs5p also localized to growing hyphal tips (see Figure 33, page 133). These results are similar to those showing that Chs3p requires Myo2p, a class V myosin, for transport to the emerging buds in *S. cerevisiae* (Santos and Synder, 1997). Moreover, in *A. nidulans*, a mold incapable of yeast budding growth, the WdChs5p ortholog, CsmA (a class V enzyme) also localizes to the hyphal tip and has an important role in the maintenance of hyphal wall integrity and polarized hyphal wall synthesis, especially under low osmotic conditions (Horiuchi *et al*., 1999, Takashita *et al*., 2002). Second, the bud tip FITC signal for WdChs5p was not observed in either the *wdcdc3*Δ*-1* or the *wdcdc10*Δ*-1* septin mutant strains (Figure 29D and E). Taken together, these results strongly suggest that a normal septin complex is crucial for WdChs5p localization at sites of polarized cell growth where WdChs5p is required to deposit chitin

Figure 33. WdChs5p localization during hyphal cell development. The wild type cells (A-F, $1x10^7$ cells/ml) grown at 26°C were transferred into the pre-warmed 37°C YPD broth, sampled after 24 h and then treated as above except that after staining with the FITC-conjugated anti- rabbit goat antibody. Bars equal 10 μm.

Figure 33



in order to maintain cell wall integrity at 37°C.

Interestingly, the localization of WdChs5p was not inhibited in the presence of nocodazole (Figure 30), which as reported previously inhibited mitosis but not bud formation (Jacobs and Szaniszlo, 1982). In contrast, in the presence of latrunculin, WdChs5p did not localize to growing bud tips, but localized aberrantly in bud necks. Moreover, when the cells were exposed to latrunculin for periods longer than 30 min, no signals for WdChs5p were observed (Figure 31). These results indicate that WdChs5p is transported to polarized cell sites through an actin based and not a microtubule based transport mechanism and that actin may also have a role in maintaining the stability of WdChs5p. The latter possibility is consistent with previous results showing that in *A. nidulans* CsmA directly binds to the actin cytoskeleton in hyphal tips (Takeshita et al., 2005; Takeshita et al., 2006).

D. WdCdc3p and WdCdc11p are required for *W. dermatitidis* conidiogenesis.

In *S. cerevisiae*, septins, and especially Spr3p and Spr28p, are important for sexual sporulation (Ozsarac *et al*., 1995; De Virgilio *et al*., 1996**)**. Also in *A. nidulans*, AspB plays an important role in hyphal branching and asexual conidiogenesis (Westfall and Momany, 2002). On PDA, a hyphal and spore induction medium, none of the chitin synthase mutants or chitin synthase *wdcdc10*Δ*-1* double mutants displayed defects in hyphal growth or conidial development (Figure 32 F-M). These results suggest that some chitin synthase functions in *W. dermatitidis* are redundant in this biological process, or alternatively, that this fungus has additional chitin synthases that might overlap with the function of the chitin synthases investigated. However, no conidium production was

observed in *wdcdc3*Δ-*1* or *wdcdc11*Δ-*1*, even though their hyphal development appeared to be normal (Figure 32B and C). In contrast, and compared to the WT strain (Figure 31A), hyphal growth and asexual spore development appeared normal in the *wdcdc10*Δ-*1* and *wdcdc12*Δ-*1* mutant strains (Figure 32D and E). These results suggest that *WdCDC3* and *WdCDC11* have a unique role in conidiogenesis in *W. dermatitidis*, most likely because a budding process is involved (Oujezdsky *et al*., 1974)

In summary, I successfully cloned and characterized four septin genes of *W. dermatitidis* and demonstrated genetic interactions between *WdCDC10* and three chitin synthase genes. I also provided the first evidence that septins and actin are required for the localization of WdChs5p, the only chitin synthase required for *W. dermatitidis* viability at high temperature and thus pathogenicity. My results further suggest that the pathways leading to the interactions detected between the septin complex and the chitin syntheses may represent additional attractive targets for development of anti-fungal drugs with usefulness in the treatment of diseases caused by fungi having considerably more varied types of chitin synthases than those of *S. cerevisiae* and *C. albicans.*

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Publications:

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